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ORIGINAL ARTICLE

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Immunohistochemical examination of skin wounds with antibodies against alpha-1-antichymotrypsin, alpha-2-macroglobulin and lysozyme

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Abstract The distribution of the proteinase inhibitors alpha-1-antichymotrypsin (α1-act), alpha-2-macroglobulin (α-2-m) and lysozyme was analysed immunohistochemically in 27 intravitally acquired wounds, 3 postmortem skin lacerations and 9 specimens of undamaged skin. Intravitally acquired wounds demonstrated distinct positive reactions for all antibodies examined (α-1-act 66.6%; α-2-m 51.9%; lysozyme 25.9%). However the undamaged skin margins opposite the wound margins also gave positive reactions (α-1-act 51.8%; α-2-m 37.0%; lysozyme 25.9%). Nearly half of the control cases (specimens of undamaged skin) exhibited weak positive reactions for all 3 antibodies. These could be easily distinguished from the strong positive reactions observed in intravitally acquired wounds. False positive reactions were observed due to contamination resulting from contact with serum components, in cases of advanced autolysis of specimens, and as a result of fixation and drying artefacts. Even though immunohistochemical studies of α-1-act, α-2-m and lysozyme give some indications concerning wound vitality, they cannot be considered as proof because irrefutable differentiation of true positive and false positive reactions is not possible in all cases.

Key words Alpha-1-antichymotrypsin · Alpha-2-macroglobulin · Lysozyme · Immunohistochemistry Wound age

Zusammenfassung Die Verteilung der Proteinasehemmer alpha-1-Antichymotrypsin (α-1-ACT) and alpha-2-Makroglobulin (α-2-M) sowie die Verteilung von Lysozym wurde in 27 intravitalen Hautwunden, drei postmortalen Hautwunden und in neun Entnahmen unverletzter Haut immunhistochemisch untersucht. Die intravitalen Wundränder zeigten mit allen untersuchten Antikörpern deutliche positive Reaktionen (α-1-ACT 66,6%; α-2-M 51,9%; Lysozym 25,9%). Der gegenüberliegende, vital unverletzte Hautrand wies jedoch bei diesen Entnahmen ebenfalls häufig schwache Reaktionen auf (α-1-ACT 51,8%; α-2-M 37,0%; Lysozym 25,9%). In nicht wenigen Fällen war diese Reaktion jedoch so stark, daß der vital unverletzte und erst durch die Hautprobenentnahme postmortal entstandene Schnittrand von der vital entstandenen Wunde nicht zu unterscheiden war (α-1-act 22,2%; α-2-M 29,6%, Lysozym 59,2%). In den Kontrollfällen (Entnahmen unverletzter Haut) fanden sich mit allen drei Antikörpern in nahezu der Hälfte der Fälle geringe positive Reationen, die jedoch aufgrund der Intensität nicht mit deutlich positiv reagierenden Hautwunden verwechselt werden konnten. Als Ursache der falsch positiven Farbreaktionen kommen artefizielle Verunreinigungen infolge Kontakts mit Serumbestandteilen, fortgeschrittene Autolyse des Präparates, Einflüsse der Fixierung und Vertrocknungen der Hauträndern in Betracht. Die immunhistochemische Untersuchung von α-1-ACT, α-2-M und Lysozym ergibt zwar häufig Hinweis auf Vitalität einer Wunde, kann diese jedoch nicht beweisen, da eine sichere Differenzierung von richtig- bzw. falsch-positiven Reaktionen nicht in allen Fällen gelingt.

Schlüsselwörter Alpha-1-Antichymotrypsin · Alpha-2-Makroglobulin · Lysozym · Immunhistochemie Wundalter

Introduction

The assessment of wound age often plays a decisive role in forensic medicine. Since the advent of immunhistochemical examination of paraffin-embedded tissues, many studies have been published using various antibodies (Oehmichen et al. [8]; Betz et al. [2] and Fechner et al. [4]). Oehmichen reported experiments with antibodies against proteinase inhibitors and lysozyme. Proteinase inhibitors regulate the activity of proteolytic enzymes and because of their role in wound healing an immunohisto-

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chemical reaction is to be expected at the wound margins. Lysozyme is detectable in many inflammatory cells. Oehmichen et al. [8] considered immunohistochemical detection of proteinase inhibitors and lysozyme in wounds a phenomenon of vitality because they failed to detect these antibodies in postmortem lacerations. In this study, the same methods as used by Oehmichen et al. were performed and results were compared.

Proteinase inhibitors are a group of proteins that regulate the activity of proteolytic enzymes by the formation of reversible complexes with proteinases.

α-1-Antichymotrypsin is a proteinase inhibitor with a molecular weight of 59,000 and a plasma concentration of 49 mg/dl which demonstrates a high specific inhibitory capacity for chymotrypsin [5, 11]. It can be detected in a number of histiocytes, mast cells, neutrophils, monocytes and macrophages [1, 3, 5, 7]. Due to its role in the acute phase of wound healing [9], α-1-act can be detected as early as 30 minutes in monolayer cultures of human skin fibroblasts [12] and in intravital skin wounds after a survival time of less than 10 minutes [8].

α-2-Macroglobulin has a molecular weight of 725,000, a plasma concentration of 268 mg/dl and is a non-specific proteinase inhibitor with activity against trypsin, chymotrypsin, plasma kallikrein, thrombin and elastase [5, 11]. It can be detected in monocytes, macrophages, lymphocytes, fibroblasts and mast cells [10] and has also been observed in intravital skin wounds after a survival period of less than 10 minutes [8]. Analyses by direct tissue isoelectric focusing have also shown a significant increase in the level of α-2-m in intravital wounds, which do not occur in postmortem wounds [13].

Lysozyme is an enzyme with a molecular weight of 14,388, which is capable of digesting bacterial cell walls. It can be detected in neutrophils, eosinophils, monophages, mast cells and histiocytes [6]. It can also been found in intravital skin wounds after a survival period of more than 60 minutes [8].

Materials and methods

Specimens of skin lesions such as surgical wounds, lacerations, gunshot and stab wounds were sampled from routine autopsies in our institute. Wound ages ranged from a few seconds to 2 weeks and postmortem intervals between death and autopsy from 8 hours to 7 days. Considering the extensive trauma accompanying 15 skin lesions, we estimated a survival period of seconds to a maximum of a few minutes. In 5 skin wounds a survival period of a few hours was documented. Four skin wounds were examined after a survival period of a few days and 3 skin wounds after 1–2 weeks. Undamaged skin from 9 specimens served as a control group. Three postmortem skin wounds were obtained from cases with wounds caused by ship's screws. The specimens were fixed in 4% formaldehyde and embedded in paraffin for the preparation of 4 µm sections. These were incubated with polyclonal antibodies against α-1-act, α-2-m and lysozyme using the peroxidase-antiperoxidase (PAP) method (Dako) as described by Oehmichen et al. [8]. DAB Chromogen (Dako Code S 3000) was used for staining. As a positive control we studied the staining of mast cells in the specimens. For the negative control of specificity, we used the proteinase inhibitors supplied by Prof. Dr. N. Heimburger Behring Werke Marburg (α-1-act: K-Nr. 041 181/22 382; α-2-m: K-Nr. 730604).

Results

α-1-act

In 18 cases (66.6%) the intravital wound margins were stained more intensively than the margins caused by postmortem excision of the specimens. From the remaining 9 cases (33.3%) 6 (22.2%) gave positive staining of similar intensity in both the wound and the postmortem skin margins. This group included survival times of less than 30 minutes and more than 24 hours (stab wounds and lacerations). We obtained no staining in the tissue specimens in 3 cases (11.1%) of extensive trauma where survival times were less than a few minutes. In 14 cases (51.8%) the necropsied skin margins demonstrated a weak positive reaction. When compared to reactions observed at the wound margins these necropsy-associated reactions were less pronounced in all cases. No staining was observed in 5 of the 9 control cases. Minimal positive staining of the entire sample was detected in 2 cases, whereas the remaining 2 samples exhibited more intensive staining at the excision margins when compared with margins produced after fixation with formaldehyde.

Two of the lesions of postmortem origin demonstrated more intensive staining in comparism to the necropsy margins. In one case weak positive staining of collagen was observed in the entire sample.

α-2-m

The intravital wound margins stained more intensively than the necropsy margins in only 14 cases (51.9%). The remaining 48.1% were composed of 8 cases (29.6%) with positive staining of similar intensity at the wound and necropsy margins. This group consisted of survival periods ranging from a few seconds to several days (stab wounds and lacerations). In 5 cases (18.5%) survival times were estimated to be less than a few minutes and we obtained no staining of the tissue. Weak staining of the necropsy margins was observed in 10 cases (37%). In all cases the reactions were weaker than the reaction at the wound margins but of the 9 control cases 5 showed no positive staining reaction. Minimal positive staining of the entire collagen tissue was observed in 3 cases. One case demonstrated more intensive staining of a necropsy margin when compared to a margin created after fixation.

One postmortem wound demonstrated more intense staining when compared with the staining of its necropsy margin and 2 postmortem wounds revealed no staining reaction.

Lysozyme

Intravital wound margins stained more intensively than the necropsy margins in only 7 cases (25.9%), which in our study included survival times of less than a few minutes. False positive results of similar intensity at the

Table 1 Material of skin lesions

Case	Wound	Localisation	Survival time	Staining reaction		
				a-1-act	a-2-m	Lysozyme
1	Laceration	Arm	Sec–min	W>E	W>E	W>E
2	Laceration	Hand	Sec–min	W>E	W>E	W>E
3	Laceration	Leg	Sec–min	W>E	W>E	W<E
4	Laceration	Leg	Sec–min	Negative	Negative	W=E
5	Surgical w.	Abdomen	Days	W>E	W>E	W=E
6	Surgical w.	Abdomen	Weeks	W=E	W=E	W=E
7	Surgical w.	Neck	Weeks	W>E	W=E	W=E
8	Laceration	Leg	Sec–min	W>E	W>E	W>E
9	Control	Abdomen	–	Negative	E1>E2	E1>E2
10	Laceration	Head	Hours	W>E	W>E	W=E
11	Laceration	Leg	Sec–min	Negative	W>E	Negative
12	Stab wound	Neck	Postmort.	W>E	W>E	W<E
13	Control	Abdomen	–	E1>E2	Negative	E1>E2
14	Laceration	Abdomen	Postmort.	W>E	Negative	W>E
15	Control	Head	–	Negative	Negative	Negative
16	Surgical w.	Abdomen	Hours	W>E	W>E	Negative
17	Control	Abdomen	–	E1>E2	Negative	Negative
18	Surgical w.	Abdomen	Days	W>E	W>E	W=E
19	Laceration	Leg	Sec–min	W>E	W>E	W<E
20	Punction	Arm	Hours	W>E	W=E	W>E
21	Gunshot	Head	Hours	W>E	W>E	W>E
22	Laceration	Neck	Sec–min	W>E	Negative	W=E
23	Laceration	Leg	Sec–min	W>E	W=E	W=E
24	Laceration	Foot	Sec–min	W=E	W>E	W=E
25	Surgical w.	Abdomen	Days	W=E	W=E	W=E
26	Surgical w.	Leg	Weeks	W=E	W=E	W=E
27	Gunshot	Head	Sec–min	W=E	W>E	Negative
28	Punction	Arm	Hours	W>E	Negative	W>E
29	Laceration	Leg	Sec–min	Negative	Negative	W>E
30	Laceration	Leg	Postmort.	W>E	Negative	W>E
31	Gunshot	Head	Sec–min	W>E	Negative	Negative
32	Laceration	Arm	Sec–min	W=E	W>E	W=E
33	Gunshot	Head	Days	W>E	W=E	W=E
34	Control	Abdomen	–	Negative	Negative	Negative
35	Control	Foot	–	Negative	E1=E2	E1>E2
36	Control	Foot	–	E1=E2	E1=E2	E1>E2
37	Control	Leg	–	E1=E2	E1=E2	E1>E2
38	Laceration	Leg	Sec–min	W>E	W=E	W=E
39	Control	Dorsum	–	Negative	Negative	Negative

W, wound margin; E, excision margin; E1, margin produced at autopsy; E2, margin produced after fixation

wound and necropsy margins were found in 16 cases (59.2%). Two cases revealed a more intense reaction at the necropsy margins than at the wound margins. No staining reaction was detected in 4 cases (14.8%), three of which included survival times of less than a few minutes. In all cases examined weak staining was detected at the necropsy margins.

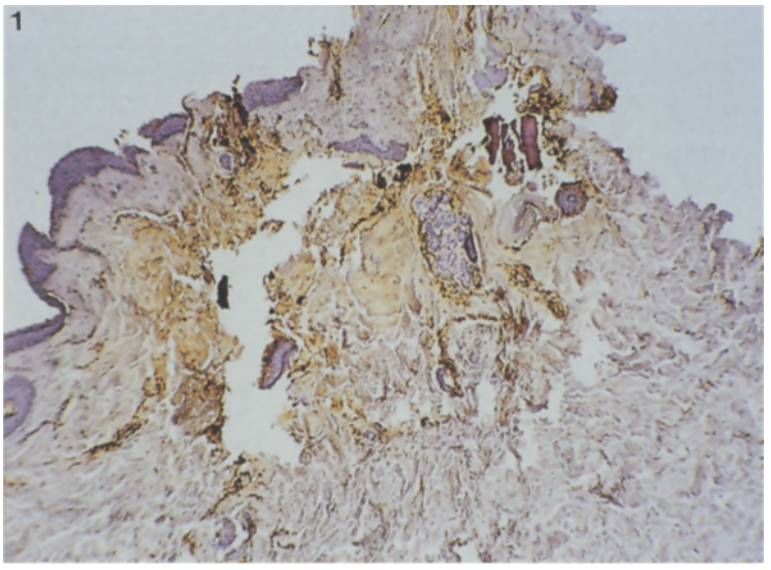
No staining reaction was detected in 4 of the 9 control cases. Weak positive staining of the necropsy margins was observed in 2 cases. The remaining 3 cases showed a weak positive reaction of the entire collagen tissue. The postmortem wounds also revealed staining reaction of the entire collagen tissue.

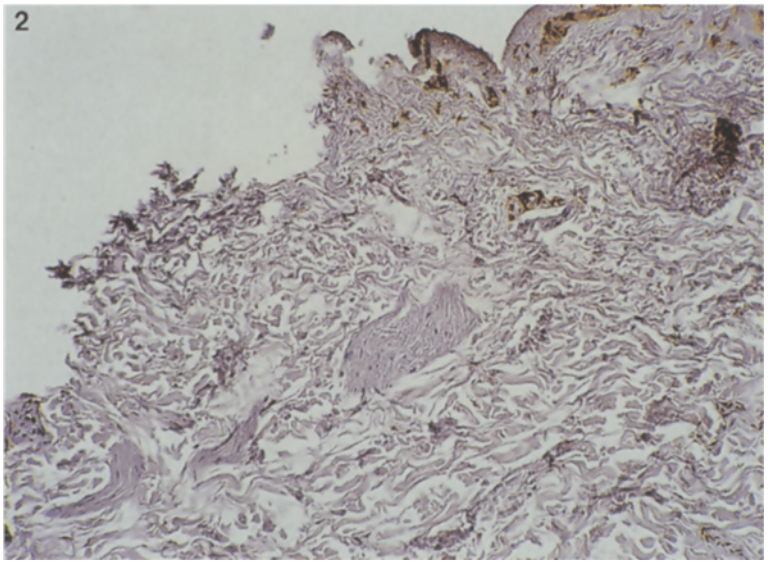
We finally compared the results of all 3 antibodies and discovered that only 25% of all examinations demonstrated the same results for all antibodies. Only in 2 cases of very short survival, did we find no conformity.

Discussion

The role of proteinase inhibitors and lysozyme in wound healing and the immunohistochemical pattern in skin wounds has been studied by Oehmichen et al. [8].

Alpha-1-antichymotrypsin (α-1-act) and alpha-2-macroglobulin (α-2-m) are 2 proteinase inhibitors, which were detected most distinctly in the study published by Oehmichen et al. but their accumulation was not observed in postmortem wounds. Analyses of proteinase inhibitors by direct tissue isoelectric focusing have also shown an in-





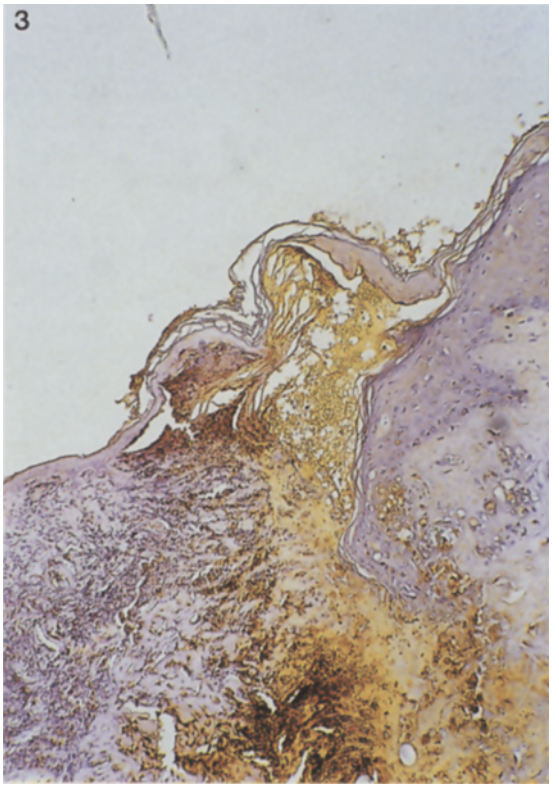


Fig. 1 Vital skin wound (traffic accident; survival time of a few minutes): positive α-1-act staining in the surrounding of a defect in the subcutis

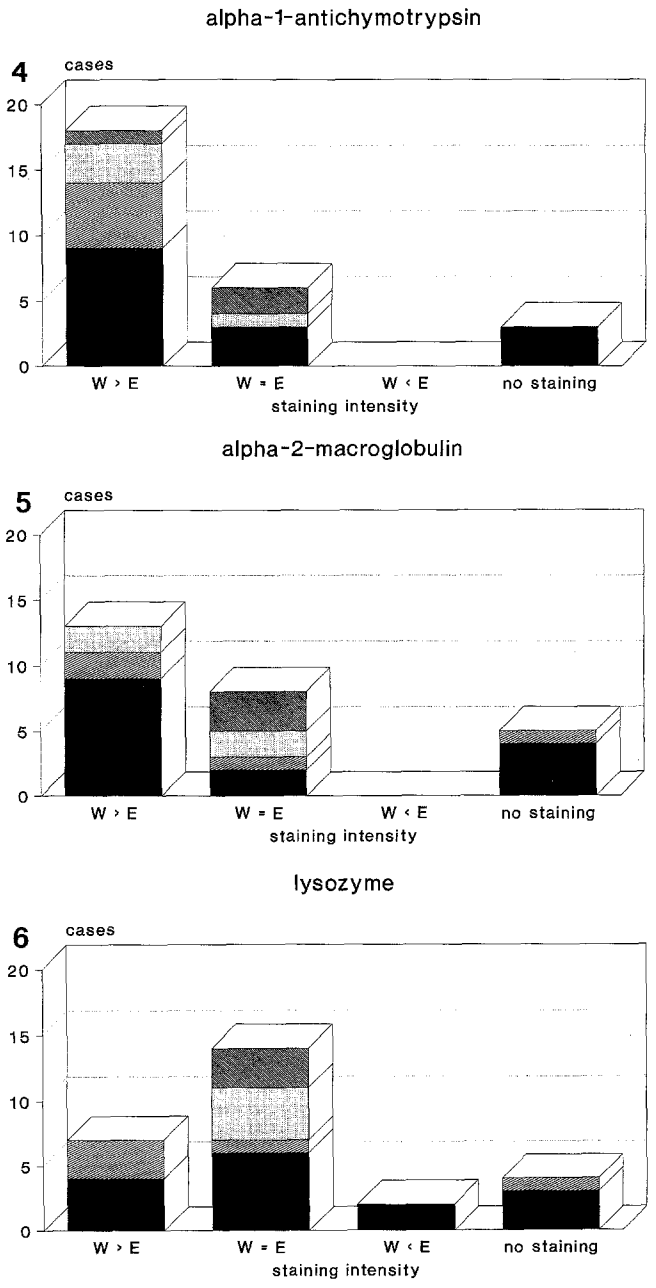
Fig. 2 Necropsy associated margin (opposite to the wound margin, same specimen as Fig.1.): no staining of the subcutis at the intravitally undamaged skin margin, positive staining reactions only in some histiocytes, mast cell or inflammatory cells

Fig. 3 Vital skin injection (due to medical treatment, survival time of a few days): positive α-1-act staining in epidermis and subcutis surrounding the injection

crease in their levels in intravital wounds, which do not occur postmortem [13]. In contrast to these results, we found false positive staining reactions in postmortem wounds. In some cases, this reaction was so intense that it was impossible to distinguish between intravitally aquired wounds and those produced by excision of specimens postmortem (α-1-act 22.2%; α-2-m 29.6%; lysozyme 59.2%). Since these proteins also occur in serum these false positive results in vitally uninjured skin can be due to postmortal "wash-in-effects" or can be explained by marginal drying artefacts as discussed in the case of fibronectin by Betz et al. [2]. In the case of injures caused by ship's screws, we also observed autolytic alterations due to a prolonged postmortem interval. We therefore conclude that the length of the postmortem interval can influence staining patterns. This may be due to postmortem outward diffusion of proteinase inhibitors and lysozyme in perished cells. The type of medium used and the length of sample fixation can also lead to changes in antigen structure and thus bring about false positive results.

Pure proteinase inhibitors were used to test the specificity of the antibodies. In these cases staining of samples was suppressed in all cases and thus non-specific binding could be excluded. In addition to false positive reactions, we also detected false negative results in some cases (α-1-act 11.1%; α-2-m 18.5%; lysozyme 14.8%). This could be explained by very short survival periods in nearly all cases. All of the results were repetive reproducible in the same samples. The best results were achieved using antibodies against α-1-act, whereas antibodies against lysozyme revealed a high number of false positive results.

Immunohistochemical investigations regarding wound age often play a decisive role in forensic medicine. Our results show that a definite differentiation between intravital and postmortem wounds is not possible in all cases. Further investigations are essential to elucidate the influ-



Figs. 4–6 Comparison of staining intensity of wound margin (W) and excision margin (E). Survival time: (■) sec-min; (▨) hours; (▩) days; (▧) weeks

ence of the postmortem interval and artefacts associated with fixation.

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