Z. Rechtsmedizin · J. Legal Med. 67, 313—318 (1970)

Early Enzyme Changes in Skin Wounds Demonstrated by Isoelectric Focusing in Polyacrylamide Gel

R. Jarecki, P. Pogacar, G. Günther and H. Klein
Institut für Gerichtliche Medizin der Universität Heidelberg

Received June 20, 1970

Summary. Esterase patterns in vital skin wound extracts were observed and compared to those seen in normal skin. Employing the relatively simple method of isoelectric focusing in polyacrylamide gel, it was demonstrated that three, consecutively appearing, characteristic changes were visible in the esterase zymograms of vital (pre-mortally) injured skin as compared with skin which was uninjured or post-mortally damaged. One of these characteristics (characteristic "a"), since it is found only in wound reactions and not in uninjured skin, may represent an enzyme specifically produced during the wound reaction process. All three features normally appeared within 30 min of trauma and the first change within 5 min, even though it had been generally assumed that vital reactions, i.e. wound reactions, could be demonstrated only after a longer period of time. It was possible therefore to determine victim survival time and distinguish between pre- and post-mortal wounds. Also the isoelectric focusing of enzymes apparently gives a more efficient pattern band separation than previous methods.

Key-Words: Skinwounds—Isoenzyme—Survival time—Isoelectric focusing—Vital reactions—Wounds.

Zusammenfassung. Durch isoelektrische Fokussierung der Extrakte aus vital verletzter Haut wurden die α-Naphthylacetat-spaltenden Enzyme aufgetrennt und mit gleichartig hergestellten Extrakten unverletzter Haut desselben Menschen verglichen. Es konnten 3 nacheinander auftretende Merkmale im Esterasemuster der verletzten Haut festgestellt werden. Die mit „a", „b" und „c" bezeichneten Unterschiede im Esterasemuster sind für die frühen Wundreaktionen kennzeichnend. Das Merkmal „a" ist eine nur in vitalen Hautwunden nachweisbare Esterase-Fraktion, die innerhalb 5 min nach der Wundsetzung auftritt. Die Merkmale „b" und „c" erscheinen nach dem Merkmal „a" innerhalb 30 min nach der Verletzung. Der Nachweis von „a", „b", „c" in einer Wunde erlaubt eine Beurteilung der Überlebenszeit und ermöglicht außerdem eine Unterscheidung von vitalen und postmortalen Wunden. Die isoelektrische Fokussierung von Enzymen scheint leistungsfähiger zu sein als die bisher zur Kennzeichnung von Wundenzymen angewandten histoenzymatischen und elektrophoretischen Methoden.

Enzyme changes in wounds have been the subject of recent histochemical [1–6], dermatological [7, 8], surgical [9], and forensic [10–15] investigations. The recent papers [12, 13] in particular, describe a determination of enzymatic wound reactions of above 10 min survival time. The characteristics were limited however to an increase or decrease of the skin enzyme activity which could not always be exactly determined and therefore did not permit correct evaluation of survival time.

Modifying and applying the relatively simple method of isoelectric focusing in polyacrylamide gel, developed by Svensson and Vesterberg [16–20], we have found distinct and well demonstrable differences between zymogram patterns of vital and post-mortal skin wounds. These differences took place within minutes after

vital skin injury. The changes occurred in the α-naphthylacetate-staining non-specific esterases seen as distinct bands of the zymogram. This would seem to indicate a rapid change in the enzyme metabolism of the damaged cell. The constant sequential appearance of the wound characteristics seems to permit the determination of victim survival time. In addition to this, a wound specific esterase band was detected.

Methods and Materials

32 morphologically different skin wounds from 22 autopsies were studied and, in each case, the skin wounds were compared with undamaged skin from the same cadaver. Skin samples were obtained as soon as possible after death, the latest being taken within 48 h, and stored at −15° C until used. They were then freed of subcutaneous fat and 600 mg of each sample chopped into small pieces, added to 3 ml TRIS phosphate buffer (pH 5), homogenized for 1 min at 0° C, allowed to stand for 24 h at 4° C and then, immediately before using, centrifuged for 20 min at 3600 r.p.m. 100 µl of the clear supernatant was used for each tube sample.

Any apparatus which is suitable for disc electrophoresis may also be used for the isoelectric focusing. The method of Dale and Latner [21] was modified for our purposes in the following way: 8 tubes, 80 mm in length, 5 mm in diameter and closed at one end with a rubber cap, were filled with Ampholine-containing polyacrylamide gel solution (Solution I) to 1 cm below the upper end and photopolymerized with four 75 Watt lamps for 90 min. 100 µl of skin extract and 200 µl of a 40% sucrose solution were transferred into the open upper end of each tube. The tubes were then inserted through the rubberlined openings between the two different solution-containing compartments of the disc electrophoresis apparatus and electrofocused for 2 h at 250 V and approximately 35 mA. The current falls to about 1 mA during the run of 2 h. 2% ethanolamine was used as the cathode solution and 1.5% phosphoric acid as the anode solution. Current was supplied by a Beckman Spinko "Duostat D2". After focusing, the gels were incubated for 1 h at 37° C in the α-naphthylacetate staining solution (Solution II) and then fixed with 10% acetic acid.

Solution I:
1.5 ml 0.8% (v/v) N,N,N′,N′ tetramethylendiamine (TEMED) solution
1.5 ml 0.004% riboflavin solution
3 ml 28% Cyanogum solution[1] (6% methylenbisacrylamide/acrylamide)
0.75 ml 0.001% bromphenol blue solution
3 ml 40% sucrose solution
0.3 ml Ampholine[2] (40% ampholytes) pH range 3–10
2 ml distilled water.

Solution II:
20 mg α-naphthylacetate, 40 mg Fast Red Tr } dissolved in 1 ml acetone
50 ml 0.04 mol. TRIS HCl buffer (4.8 gm TRIS + 34 ml 1 N HCl ad 1000 ml H_2O).

Results

During isoelectric focusing the esterases, being proteins, distribute themselves along the length of the Ampholine-containing polyacrylamide gel tubes according to their isoelectric points only and therefore do not correspond to patterns seen in regular electrophoresis procedures. The esterase patterns of skin from the same cadaver were identical and reproducible, different cadavers however, often showed a slightly different basic pattern.

1 Cyanamid, U.S.A.
2 LKB, Sweden.

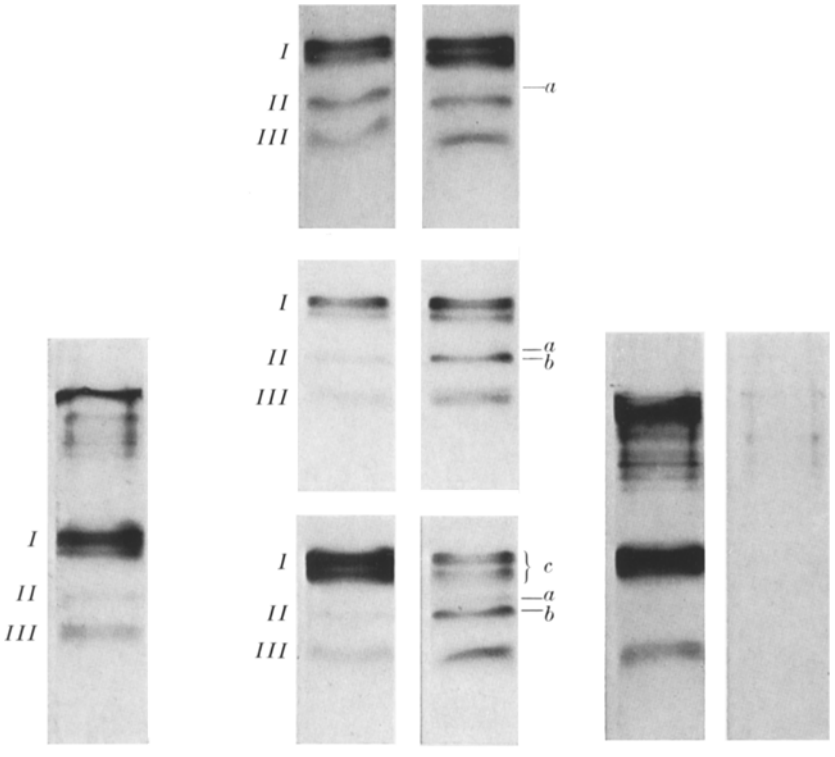

Fig. 1 Fig. 2 A—C Fig. 3

Fig. 1. Pattern of normal uninjured skin *I*, *II*, *III*: Groups

Fig. 2 A–C. Left = uninjured skin; right = injured skin. *I*, *II*, *III*: Groups. *a*, *b*, *c*: Characteristics

Fig. 3. Electric shock injury (notice lack of esterase activity in the injured skin)

Staining revealed 15 esterase bands, 6 or 7 of which were seen in the alkaline and neutral zones and were not useful in evaluating the vital reactions (Fig. 1). The enzyme changes which were found to be characteristic of wound reactions occurred however, among the remaining esterase bands appearing in the acid region, in the lower part of the tube. These bands are arbitrarily divided into Groups I, II and III (see Fig. 1).

Group I consists of two closely adjacent, strongly staining bands which are always present and are bordered inconsistently by up to two weakly staining bands just above and/or just below the two primary bands.

Group II is a single band which may or may not be intensively stained but which, in contrast to the other groups, can be inhibited by eserine and cannot be stained with naphthyl-butyrate.

Group III consists of 2 or 3 bands lying in close proximity to each other and is part of the albumin fraction.

Table

Esterase pattern	Survival time in minutes		
	up to 5 min (estimated)	5 to 30 min (estimated)	over 30 min
No changes	x	xx	
Characteristic "a"		xxx	xx
Characteristics "a" and "b"		xxx	x (hematoma)
Characteristics "a", "b" and "c"		xx	xxxxxx

The pre-mortally injured skin patterns demonstrate up to 3 changes which differentiate them from the normal skin patterns as follows:

a) The appearance of a new band above the band of Group II which we have designated as the "a" band (Fig. 2A).

b) An intensification of the Group II band (Fig. 2B).

c) A lessening in intensity of the Group I complex (Fig. 2C).

These characteristics appear in sequence related to the victim's survival time with "a" indicating the shortest and "c" the longest period of survival. Only characteristic "a" may appear by itself, whereas "b" appears only with "a", and "c" only with "a" and "b".

The wound zymograms of 22 cadavers demonstrated the following: 4 wounds — no changes; 5 wounds — characteristic "a" only; 4 wounds — characteristics "a" and "b"; 8 wounds — all three ("a", "b" and "c"). The table below illustrates the wound esterase patterns dependent upon the victims' survival time after injury. One wound zymogram, an electric shock burn, was atypical (Fig. 3). One which showed no features of wound reaction (a, b, or c) was from a case of barbiturate poisoning and the wound was of post-mortal origin.

Discussion

The preceeding data led to the conclusion that significant esterase pattern changes occurred as a result of wound reaction and that these changes could be seen shortly after origin of the pre-mortal wound. The reactions (and changes) were missing only if death took place immediately after injury or if the blood supply to the wounded area was interrupted very quickly. Since the timing of wounds occurring in cases in which death quickly followed injury (death usually took place at the scene of the accident) could only be estimated, an exact time table for the early changes could not be made. It can be stated however, that characteristic "a" shows as the first feature and that it appears within minutes after injury and seems to be specific for the wound reaction process. Possibly, this characteristic "a" may be related to the arylaminopeptidase activity found by Raekallio [4]. It is followed consecutively by the second characteristic (intensification of Band II) and then the third (weakening of the Group I complex), the final change taking place within 30 min after injury. This sequence may be assumed to be true since no other combination of characteristics was seen in the entire series of skin wound zymograms examined. There was only one exception

in which, possibly due to hereditary factors, neither a Group II band nor an "a" band was visualized.

Of the 11 zymograms of skin wounds of the victims who died within 30 min of injury, three showed no pattern changes (vital reactions); of the remaining 8, three demonstrated change "a", three changes "a" and "b", and two showed all changes "a", "b", and "c" (see previous Table). It could be concluded therefore, that within 30 min all three characteristics of wound reactions would appear. Among the 9 wounds with which the victims survived more than 30 min there were 2 instances of the appearance of the "a" band only. One of these was a 9-day-old abrasion wound and the presence of only the "a" band could indicate that the healing process had progressed so far as to reverse the other two signs. The other case was an operative wound and the enzyme reactions possibly could have been influenced by the operation, operative or post-operative wound treatment. The remaining wounds, as was expected, produced patterns in which all the characteristic changes were visible.

Zymograms of hematomas indicated that in this type of injury there is a delay in the enzyme reaction — a longer survival time was necessary for the appearance of the vital reaction pattern.

The zymogram patterns obtained from skin injuries cannot be attributed to serum, whole blood, or subcutaneous fat contamination which may be found in the wound tissue sample since, taken alone, these substances show none of the wound reaction characteristics. Comparisons of pre-mortal with post-mortally damaged skin showed that no enzyme reactions took place in tissue damaged post-mortally, ruling out the possibility of post-mortal autolysis accounting for the zymogram changes.

References

1. Raekallio, J.: Enzymes histochemically demonstrable in the earliest phase of wound healing. Nature (Lond.) **188**, 234–235 (1960).
2. — Lindfors, R., Elfving, G., Hästbacka, J. E., Puittinen, J.: Histochemical observations on wound healing in denervated and healthy rat skin. Acta path. microbiol. scand. **62**, 53 (1964).
3. — Die Altersbestimmung mechanisch bedingter Hautwunden mit enzymhistochemischen Methoden. Arbeitsmethoden der medizinischen und naturwissenschaftlichen Kriminalistik, B. 3. Lübeck: Max Schmidt-Römhild 1965.
4. — Mäkinen, P. L.: Biochemical and histochemical observations on ammopeptidase activity in early wounds healing. Nature (Lond.) **213**, 1037 (1967).
5. Montagna, W.: Histology and cytochemistry of human skin. IX. The distribution of non-specific esterases. J. biophys. biochem. Cytol. **1**, No. 1, 13—16 (1955).
6. Gomory, G.: The histochemistry of esterases. Int. Rev. Cytol. **1**, 323 (1952).
7. Matoltsy, A. G., Matoltsy, M. N.: A study of the soluble proteins of normal and pathologic horny tissues by a modified disc-electrophoresis technique. J. invest. Derm. **41**, 255 (1963).
8. Marghescu, S., Braun-Falco, O.: Über Esterasen-Isoenzyme in der Hornschicht bei Hautgesunden sowie bei Psoriasis, Ekzem und Ichthyosis. Enzymelektrophoretische Untersuchungen an Schuppenextrakten. Arch. klin. exp. Derm. **224**, 42—47 (1966).
9. Lindner, J.: Die Morphologie der Wundheilung. Langenbecks Arch. klin. Chir. **301**, 39—66 (1962).
10. Pioch, W.: Zur vitalen Reaktion von Brandwunden der Haut. Konventionelle und histochemische Untersuchungen. Vortr. 42. Tag. Dtsch. Ges. gerichtl. Med., München (1963). Dtsch. Z. ges. gerichtl. Med. **55**, 242 (1964).

11. Mäkinen, P. L., Raekallio, J.: Studies on the formation of ammopeptidase-like enzymes on regenerating wound tissue. Acta chem. scand. **21**, 761 (1967).
12. Pioch, W.: Epidermale Esterase-Aktivität als Beweis der vitalen Einwirkung von stumpfer Gewalt. Beitr. gerichtl. Med. **25**, 136—145 (1967).
13. Mäkinen, P. L., Raekallio, J.: Fractionation and characterization of arylamidases in regenerating wound tissue. Acta chem. scand. **22**, 597 (1968).
14. Raekallio, J., Mäkinen, P. L.: On the origin of wound arylaminopeptidases. Experientia (Basel) **25**, 929 (1969).
15. Jarecki, R., Klein, H.: Zur Unterscheidung vitaler und postmortaler Wunden durch Bestimmung der Esterasemuster der Haut. Dtsch. Z. ges. gerichtl. Med. **66**, 161—169 (1970).
16. Svensson, H.: Isoelectric fractionation, analysis and characterization of ampholytes in natural pH-gradients. I. The differential equation of solute concentration at a steady state and its solution for simple cases. Acta chem. scand. **15**, No. 2, 325–341 (1961).
17. — Isoelectric fractionation, analysis and characterization of ampholytes in natural pH-gradients. II. Buffering capacity and conductance of isoinic ampholytes. Acta chem. scand. **16**, 456–466 (1962).
18. — Isoelectric fractionation, analysis and characterization of ampholytes in natural pH-gradients. III. Description of apparatus for electrolysis in columns stabilized by density gradients and direct determination of isoelectric points. Arch. Biochem. **1**, 132–138 (1962).
19. Vesterberg, O., Svensson, H.: Isoelectric fractionation, analysis and characterization of ampholytes in natural pH-gradients. IV. Further studies on the resolving power in connection with separation of myoglobin. Acta chem. scand. **20**, 820–834 (1966).
20. — Isoelectric fractionation, analysis and characterization of ampholytes in natural pH-gradients. V. Separation of myoglobins and studies on their electro-chemical differences. Acta chem. scand. **21**, 206–216 (1967).
21. Dale, G., Latner, A.: Isoelectric focusing in polyacrylamide gels. Lancet **1968 I**, 847–848.

Dr. med. Richard Jarecki
Dr. rer. nat. Peter Pogacar
Dr. med. Gabriele Günther
Prof. Dr. med. Hans Klein
Institut für gerichtliche Medizin
D-6900 Heidelberg
Voss-Straße 2