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Influence of postmortem changes on immunohistochemical reactions in skin

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Abstract The influence of postmortem damage of tissues on the immunohistochemical diagnosis of wound age has not as yet been clarified. We utilized antibodies against the proteinase inhibitors α-1-antichymotrypsin and α-2-macroglobulin, fibronectin and lysozyme to study samples of skin which had been intact intravitally, but were damaged postmortem either by autolysis or compression with a surgical clamp at the time of dissection. Even in the absence of autolysis, antibodies against the proteinase inhibitors and fibronectin exhibited staining of tissue margins. Autolysis caused an increase in false positive results. In contrast, antibodies against lysozyme did not give false positive staining. There were no antigens sensitive to postmortem clamping and false positive results were not observed. Antibodies against proteinase inhibitors are not useful for the diagnosis of wound age because of a high number of false positive reactions in marginal areas. Fibronectin also showed false positive band-shaped staining patterns at the tissue margin. In addition, autolytic processes increase the number of false positives. The antibody against lysozyme is much less sensitive to autolysis and no false positive reactions were observed in our series of tests.

Key words Proteinase inhibitors · Fibronectin · Lysozyme · Immunohistochemistry · Autolysis

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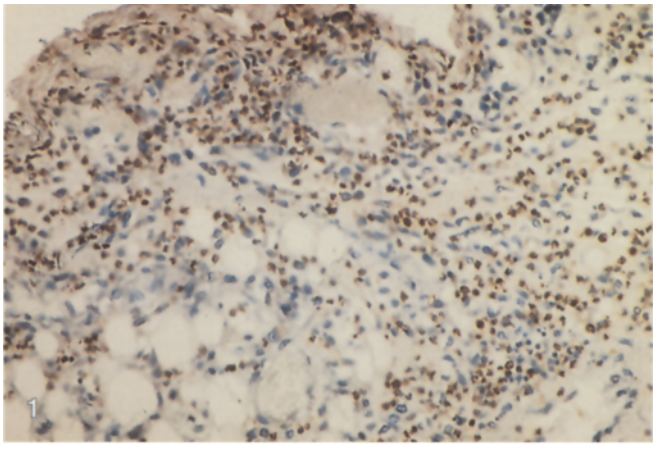
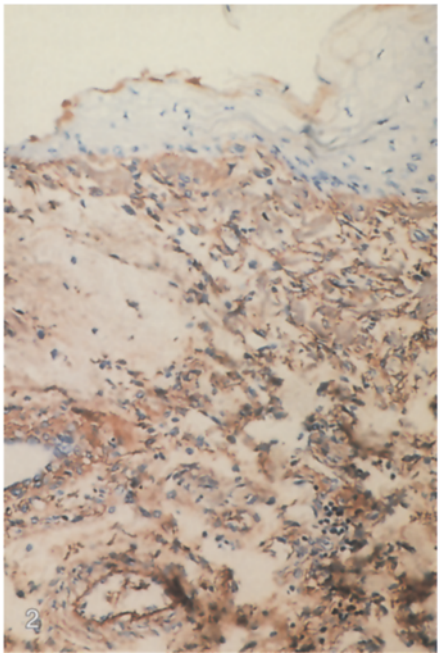
Introduction

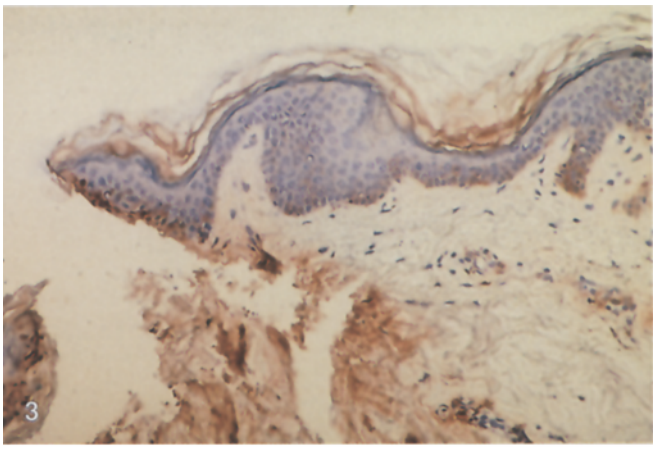
Mediators of wound healing such as fibronectin, lysozyme and the proteinase inhibitors α-1-antichymotrypsin (α-1-Act) and α-2-macroglobulin (α-2-m) have been used in immunohistochemical studies to distinguish between intravitally acquired wounds and those occurring postmortem (Grinnell et al. 1981; Viljanto et al. 1981; Oehmichen et al. 1989 a, b; Oehmichen 1990; Betz et al. 1992). However false positive reactions have been noted and may be due to specific staining reactions (e.g. specific staining of washed-in serum components) or nonspecific binding to antigens which become exposed postmortem (Viljanto et al. 1981; Betz et al. 1992, 1993a; Fieguth et al. 1994). Generally, staining in areas adjacent to necrotic or damaged cells is considered nonspecific. For example the macrophage antibody MRP 8, that is also used for wound-age estimation, shows non-specific reactions with necrotic material (Schulz-Schaeffer et al. 1996). Necrotic tissue initiates the release of lysosomal proteolytic enzymes, which change molecular structures and antigenic properties (True 1990). When studying postmortem skin specimens, the tissue is often damaged and autolysis has set in. The goal of this study was therefore to determine the influence of autolysis and mechanical postmortem tissue damage on the staining pattern of different markers.

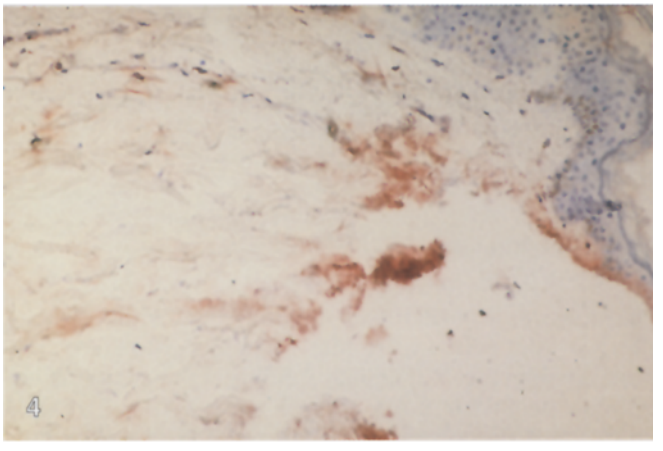
Materials and methods

The influence of postmortem tissue changes on immunohistochemical reactions using primary antibodies against α-1-Act, α-2-m, lysozyme and fibronectin were studied in more than 250 microscope slide preparations of undamaged abdominal skin that were removed during five random autopsies in our department. The postmortem interval ranged between 6 and 56 h. In every case the cadavers were stored at 4° C. None of the cadavers showed putrescence at autopsy. From each autopsy six skin specimens were taken. Of the total 30 skin specimens, 10 were fixed in formalin immediately during autopsy (day 1), 10 were fixed after storage at room temperature (day 2) and 10 were fixed after a period of 3









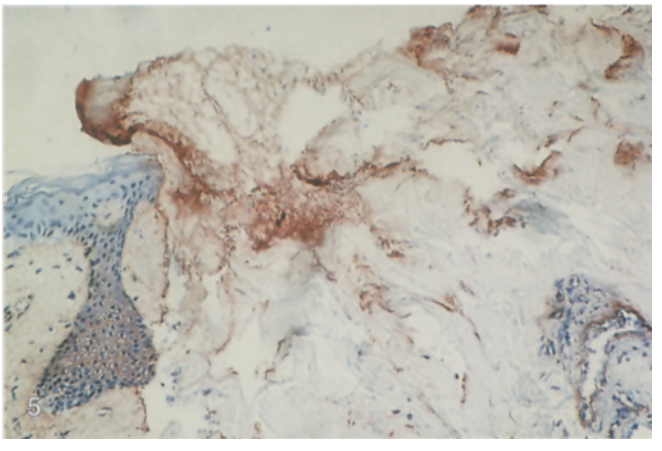


Fig. 1 Granulation tissue of the skin with lysozyme-positive inflammatory cells (Paraffin, streptavidin/biotin-peroxidase method, × 160)

Fig. 2 Granulation tissue of the skin with fibronectin-network (Paraffin, streptavidin/biotin-peroxidase method, × 160)

Fig. 3 Skin specimen (2nd day) with a non-specific marginal staining for α-1-antichymotrypsin (Paraffin, streptavidin/biotin-peroxidase method, × 160)

Fig. 4 Skin specimen (3rd day) with a non-specific marginal staining for α-2-macroglobulin (Paraffin, streptavidin/biotin-peroxidase method, × 160)

Fig. 5 5 Skin specimen (3rd day) with a non-specific marginal staining for fibronectin (Paraffin, streptavidin/biotin-peroxidase method, × 160)

days. In order to study the influence of postmortem tissue damaging, half of the samples were clamped with a surgical clamp for a short period prior to fixation. The skin specimens were fixed in 4% acid-free stabilized formaldehyde (pH 7.2) after a fixation time of 24 h and subsequently embedded in paraffin and cut in 2 µm thick sections. All of the 30 skin specimens were stained twice immunohistochemically with the four antibodies mentioned above (i. e. 240 slides) to verify correct staining results. In 95% we had similar staining results, in 5% a third staining was necessary. Negative controls were also included in which the primary antibodies were replaced by immunoglobulins of non-immunized rabbits. Granulation tissues were used as positive controls (Figs. 1 and 2).

The polyclonal antibodies against α-1-antichymotrypsin, α-2-macroglobulin, fibronectin and lysozyme (Dako, Hamburg, Germany) were detected using a streptavidin-biotin-peroxidase-complex (Immunotech, Hamburg, Germany). Fibronectin was detected after enzymatic digestion with pepsin (Hsu et al. 1981; Kirkpatrick and D'Ardenne 1984).

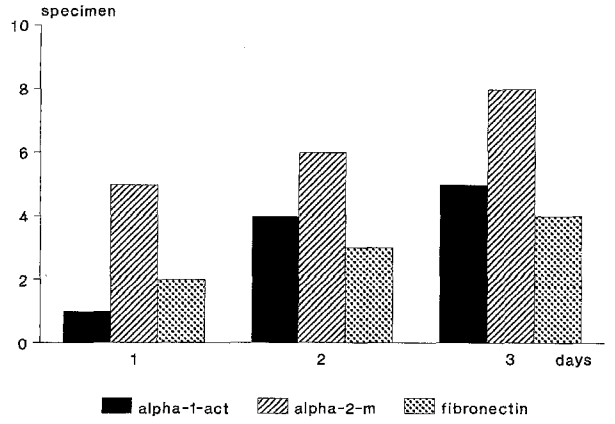


Fig. 6 Comparison of non-specific marginal staining of α-1-antichymotrypsin, α-2-macroglobulin and fibronectin depending on the length of autolysis (10 specimens/day, $n = 30$)

Results

Influence of autolysis

No increase of background staining was seen in association with the period of storage of specimens before fixation. An increase in marginal staining was noted with antibodies against α-1-Act (Figs. 3 and 6), α-2-m (Figs. 4 and 6) and fibronectin (Figs. 5 and 6). No increase was noted with lysozyme. Nonspecific marginal staining occurred mainly with α-2-Act with a high number of false positives even at day 1.

Influence of clamping

Comparing clamped with intact skin, no differences were seen in the staining pattern with any of the markers. However, a difference in staining intensity was noted due to the antibody used. Using antibodies against the proteinase inhibitors α-1-Act and α-2-m the clamped areas were blank in all cases when background staining was present. The same result was found when using high concentrations of non-immune serum. Because antibodies against lysozyme did not produce background staining, this effect was not observed. Finally, antibodies against fibronectin did not demonstrate loss of background staining at clamped regions.

Discussion

The results showed that the influence of autolysis depends on the marker used. Autolysis may cause a reduced staining intensity (Betz et al. 1995) or non-specific reactions. The occurrence of non-specific reactions when using antibodies against α-1-Act and α-2-m has been described by many authors previously (Vilijanto et al. 1981; Betz et al. 1992, 1993a; Fieguth et al. 1994). Non-specific reactions of antibodies against fibronectin were described at the margin of specimens and after putrefication. Postmortem injuries of skeletal muscle occasionally show positive fibronectin reactions at the outer surface of the sarcolemma, whereas vital staining occurs in the sarcolemma tubules (Fechner et al. 1993). Band-shaped non-specific staining should be distinguishable from vital fibronectin staining with network-like structures (Betz et al. 1993a,b). Further investigations also showed network-like structures in postmortem wounds of pigs that were induced 0–5 min after cardiac arrest (Grellner, personal communication). The interpretation of results must therefore include not only an assessment of the form and distribution of staining patterns, but must also encompass a comparison of these findings with control specimens taken from undamaged skin, especially if specimens are poorly conserved.

In clamped areas autolytic changes did not result in false positive reactions. Interestingly, the results with the proteinase inhibitors α-1-Act and α-2-m showed an opposite effect. A loss of the background staining of the samples was seen. Because clamping forces serum out of the tissues it is possible that humoral not cellular components are responsible for the staining reaction. Reid et al. (1987) also reported that delayed fixation may result in diffusion of antigen-containing components causing false interpretation of such immunohistochemical reactions. Staining of these presumed serum components did not occur in negative controls in which the primary antibody was omitted. However it was present in negative controls with high concentrations of non-immune rabbit serum. This means that not only anti-human rabbit antibody can cause specific staining but also that other antibodies of non-immunized rabbits may cause staining if they are not sufficiently diluted. False positive reactions with non-immune serum of rabbits has also been reported with reference to other antibodies (Reinhold-Richter and Renner 1991).

For this reason the evaluation of wound age is difficult, particulary if antibodies against serum components are used. We therefore recommend using controls from undamaged skin from comparable sites not only if autolysis is present but also if the specimens are severely damaged.

References

Betz P, Nerlich A, Wilske J, Tübel J, Wiest I, Penning R, Eisenmenger W (1992) Immunohistochemical localisation of fibronectin as a tool for the age determination of human skin wounds. Int J Legal Med 105:21-26

Betz P, Nerlich A, Wilske J, Tübel J, Penning R, Eisenmenger W (1993a) The immunohistochemical localisation of alpha-1-antichymotrypsin and fibronectin and its meaning for the determination of the vitality of human skin wounds. Int J Legal Med 105:223-227

Betz P, Nerlich A, Wilske J, Tübel J, Penning R, Eisenmenger W (1993b) The immunohistochemical analysis of fibronectin, collagen type III, laminin, and cytokeratin 5 in putrified skin. Forensic Sci Int 61:35-42

Betz P, Tübel J, Eisenmenger W (1995) Immunohistochemical analysis of markers for different macrophage phenotypes and their use for a forensic wound age estimation. Int J Legal Med 107:197-200

Fechner G, Bajanowski Th, Brinkmann B (1993) Immunohistochemical alterations after muscle trauma. Int J Legal Med 105: 203-207

Fieguth A, Kleemann WJ, Tröger HD (1994) Immunohistochemical examination of skin wounds with antibodies against alpha-1-antichymotrypsin, alpha-2-macroglobulin and lysozyme. Int J Legal Med 107:29-33

Grinnell F, Billingham RE, Burgess L (1981) Distribution of fibronectin during wound healing in vivo. J Invest Dermatol 76: 181-189

Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques, a comparision between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577-580

Kirkpatrick P, D'Ardenne AJ (1984) Effects of fixation and enzymatic digestion on the immunohistochemical demonstration of laminin and fibronectin in paraffin embedded tissue. J Clin Pathol 37:639-644

Oehmichen M (1990) Die Wundheilung. Theorie und Praxis der Chronomorphologie von Verletzungen in der forensischen Pathologie. Springer, Berlin Heidelberg, pp 15-16

Oehmichen M, Schmidt V, Stuka K (1989a) Freisetzung von Proteinase-Inhibitoren als vitale Reaktion im frühen posttraumatischen Intervall. Z Rechtsmed 102:461-472

Oehmichen M, Schmidt V, Stuka K (1989b) Immunhistochemischer Vitalitätsnachweis von offenen Hautwunden am Paraffinschnitt. Beitr Gerichtl Med 4:7-11

Reid WA, Branch T, Thompson WD, Kay J (1987) The effect of diffusion on the immunolocalization of antigen. Histopathology 11:1277-1284

Reinhold-Richter L, Renner H (1991) Normale Kaninchenseren. Eine immunologische und immunhistologische Studie. Zentralbl Pathol 137:66-68

True LD (1990) Diagnostic immunhistopathology. Gower Medical, New York London, p 1.3

Schulz-Schaeffer WJ, Brück W, Püschel K (1996) Macophage subtyping in the determination of age of injection sites. Int J Legal Med 109:29-33

Viljanto J, Pentinnen R, Raekallio J (1981) Fibronectin in early phases of wound healing in children. Acta Chir Scand 147:7-13