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ORIGINAL ARTICLE

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Expression of fibronectin and tenascin as a demonstration of vital reaction in rat skin and muscle

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Abstract The efficiency of immunohistochemical techniques for the diagnosis of vitality of wounds decreases for lesions occurring fairly close to death. We analyzed the expression of fibronectin (FN) and tenascin (TN) in wounds inflicted in abdominal skin of 12 adult rats. An incised injury was made at 5, 10 or 15 min before death and another at 5 min after sacrifice, and collected after 45 min. Formalin-fixed paraffin-embedded sections from a total of 36 samples (mean 1.5 per wound) were immunostained following the streptABC technique. Microscopic examination revealed a reticular pattern staining in 18 out of 20 vital samples for FN, 16 out of 20 for TN, 2 out of 16 postmortem samples for FN and 3 out of 16 for TN. Intracellular staining of muscle fibres was observed in 7 out of 20 vital and 5 out of 16 postmortem samples. FN and TN were detected in most of the vital injuries but they are not completely specific. Postmortem staining occurred in a few cases probably related to a passive extravasation of these molecules from damaged blood vessels. Reactivity of muscle fibres occurs both in vital and postmortem lesions, and is not useful in the diagnosis of vitality.

Keywords Fibronectin · Tenascin · Vital reaction · Wound healing · Immunohistochemistry

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Introduction

The determination of vitality of skin wounds constitutes a common problem for the forensic pathologist and the differentiation between vital and postmortem wounds is usually based on gross and histological studies. Ancillary techniques such as electron microscopy, biochemical methods and immunohistochemistry have also been used to aid the diagnosis (Betz et al. 1992, 1993a; Betz 1994; Vieira 1996; Ohshima 2000).

First investigations by Raekallio showed age-dependent changes in enzyme activity of injuries detectable through histochemistry, describing the existence of different zones of reactivity in relation to the wound margin (Raekallio 1967).

Immunohistochemical detection of factors that mediate the inflammatory reaction and healing process has recently received special attention, such as the different cells implied (Bonelli et al. 2003), inflammatory mediators (Piercecchi-Marti et al. 2001; Kondo et al. 2002a, 2002b) and apoptotic phenomena (Edston et al. 2002; Nakatome et al. 2002; Suárez-Peñaranda et al. 2002). Amongst them, fibronectin (FN) and p-selectin are outstanding as they have been proposed as extremely early markers of vitality in skin injuries (Viljanto et al. 1981; Betz et al. 1992; Betz 1995; Dressler et al. 1999). Previous reports defined as a positive reaction staining of the injury margin in continuity with a network of ramifying fibres (Betz et al. 1992; Betz 1995).

However these methods are more valuable when dealing with vital wounds with long survival periods and with lesions produced after long postmortem intervals. When forensic practice is confronted with lesions close to (before or after) death, the efficiency of these techniques decreases, and differential diagnosis is more difficult (Vieira 1996; Ohshima 2000).

For obvious reasons it is difficult to investigate vital vs. postmortem human injuries which have been inflicted extremely close to death and to compare them in the same individual. The objective of this present study was to carry out such a comparison performed on rats.

We have analyzed the immunohistochemical expression of FN and tenascin (TN), molecules involved in inflammatory and healing processes (Juhasz et al. 1993; Thomas et al. 1995), in vital and postmortem wounds in rat skin and subjacent skeletal muscle, inflicted on the same individuals at extremely short intervals both before and after death.

Materials and methods

Twelve narcotised male adult rats (*Rattus norvegicus*) weighting 230–297 g (mean 256 g) were used. Incised wounds were inflicted with a scalpel in the abdominal skin. The injuries measured about 1 cm in length and 0.3–0.4 cm in depth, and were inflicted 5, 10 or 15 min before death (3 groups of 4 individuals). The rats were sacrificed by intravenous injection of pentothal, which resulted in respiratory and myocardial depression. In every animal a similar injury was performed in the contralateral skin 5 min after death. All samples were collected after an interval of 45 min after death.

All animal experiments were carried out in accordance with the principles of laboratory animal care (U.S. DHEW 1985).

Transversal sections were taken from the wounds, amounting to a total of 36 samples (mean 1.5 per wound). The specimens were fixed in 4% formaldehyde, processed, embedded in paraffin and cut into 3 µ sections.

The immunostaining procedure followed the streptABC technique (Hsu et al. 1981). Tissue sections were deparaffinized and antigen retrieval was then performed with enzyme pretreatment for FN (30 -min digestion with 0.1% trypsin, at 37°C and pH 7.8); or heating for TN in 10 mmol/l, pH 6.0 citrate buffer, in an 800 W microwave oven, for 3×5 min.

Sections (two per specimen) were incubated with a purified polyclonal rabbit antibody against FN (1:2000), which has been previously reported to react with rat tissues (Dako specification sheet rabbit anti-human fibronectin Code No. A0245 Lot 097.Report No: A 0245/MN/24.10.97), and a monoclonal mouse anti-TN antibody (clone TN2, 1:500), (DAKO, Glostrup, Denmark) for 1 h at room temperature. Biotinylated anti-mouse goat antibody plus blocking 1% goat serum was then applied for 30 min. After inhibition of endogenous peroxidase activity (3% hydrogen peroxide aqueous solution for 10 min), the slides were incubated with the streptavidin-biotin-peroxidase complex for 30 min.

Development was carried out using diaminobenzidine and 3% hydrogen peroxide for 10 min. Finally the sections were counterstained with hematoxylin, dehydrated and mounted in Eukitt.

Staining of some specimens without the inclusion of the primary antibody, which was replaced by phosphate-buffered saline (PBS) (pH 7.6) was performed for negative controls.

Slides were evaluated by light microscopy. After checking the positivity of internal controls, staining of the wound edge and adjacent tissue was investigated. For the analysis of the groups of cases, differences in proportions were tested by using the χ^2 test. Differences were considered statistically significant when $p<0.05$.

Results

Normal skin

Expression of FN and TN was detected in basal membranes, vascular endothelium and fibroblasts (Fig. 1). This staining was considered as the internal positive control. Occasional reactivity was observed in sebaceous glands and adipocytes.

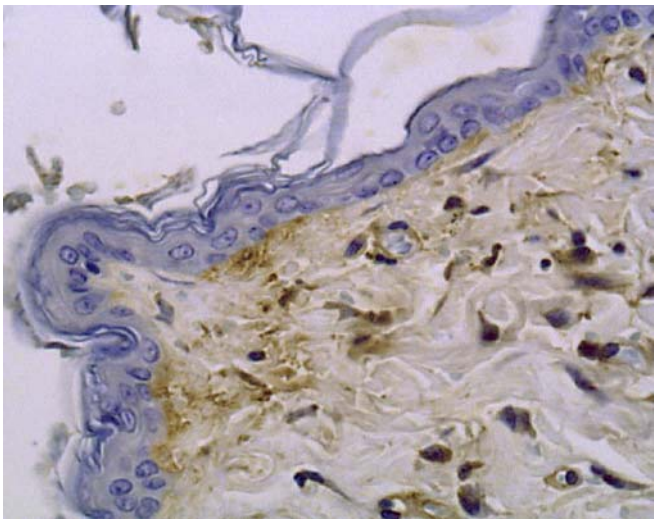

Fig. 1 Positive staining control: expression of FN in epidermal basal membrane and dermal fibroblasts (×400)

Table 1 FN and TN reticular staining in vital and postmortem wounds

Type of wound	No. rats	Total of samples	FN: reactive samples	TN: reactive samples
Vital, 5 min.	4	5	80%	60%
Vital, 10 min.	4	6	83%	67%
Vital, 15 min.	4	9	100%	100%
Postmortem	12	16	12.5%	18.7%

Wounds

A reticular staining was observed in 18 out of 20 (90%) vital samples for FN and 16 out of 20 (80%) vital samples for TN. With regard to the postmortem injuries, this pattern of reaction was found in 2 out of 16 (12.5%) samples for FN and 3 out of 16 (18.7%) samples for TN. The prevalence of this reaction according to the kind and age of wound is detailed in Table 1.

The staining pattern was similar for both molecules in each specimen, but the intensity for FN was stronger than that for TN. Reticular staining originated from the edge of the injury towards the deep dermis and, mainly, subcutaneous tissue, aponeurosis and interstitially in the muscle tissue (Fig. 2).

In those samples with an obvious component of hemorrhage the staining was enhanced.

The reticular pattern was therefore more prevalent in vital rather than in postmortem wounds and this difference was statistically significant ($p<0.001$ for FN and $p=0.001$ for TN).

This pattern was not absolutely specific as it was observed in postmortem wounds (12.5% for FN, and 18.7% for TN) (Fig. 3). These latter consisted of 5 samples (2 for FN and 3 for TN) from 2 individuals, where vital wounds (aged 15 and 5 min) also showed expression of both substances in all of the samples (Table 2).

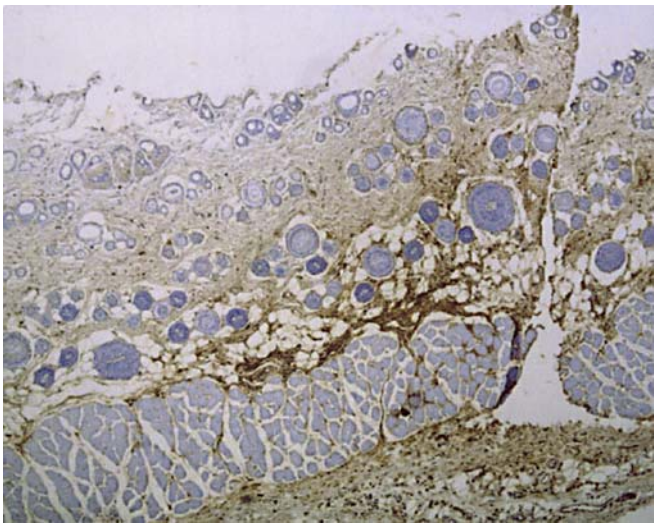

Fig. 2 Vital wound: immunoreactivity for FN: network staining from the edge of the injury through deep dermis, subcutaneous tissue and superficial muscle (×40)

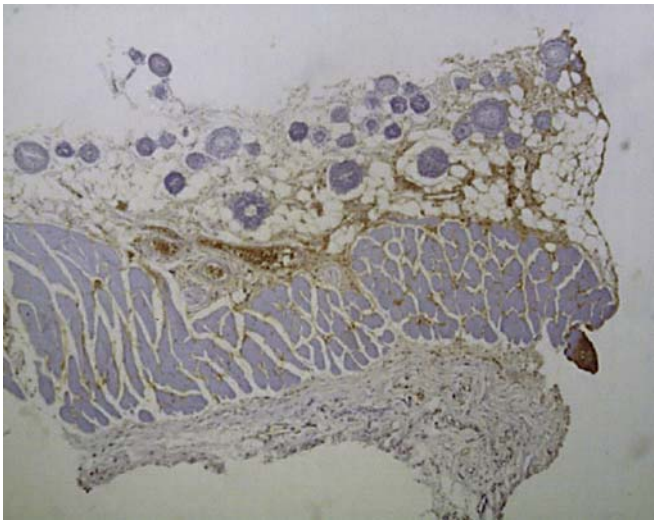

Fig. 3 Postmortem wound: edge and reticular-pattern dermal staining for FN (×40)

Table 2 Reactive postmortem cases: comparison with vital samples from the same rats

Rat	Vital wound with reaction	Postmortem expression	Vital samples: reactive/total	Postmortem samples: reactive/total
1	5 min	FN	2/2	2/2
		TN	2/2	2/2
2	15 min	TN	2/2	1/2

Apart from interstitial staining, intracellular reactivity of some muscle fibers (Fig. 4) was observed in occasional vital and postmortem wounds (Table 3). It was present in 7 out of 20 vital samples (35%) and in 5 out of 16 postmortem samples (31.25%). This difference was not statistically significant (p=0.549).

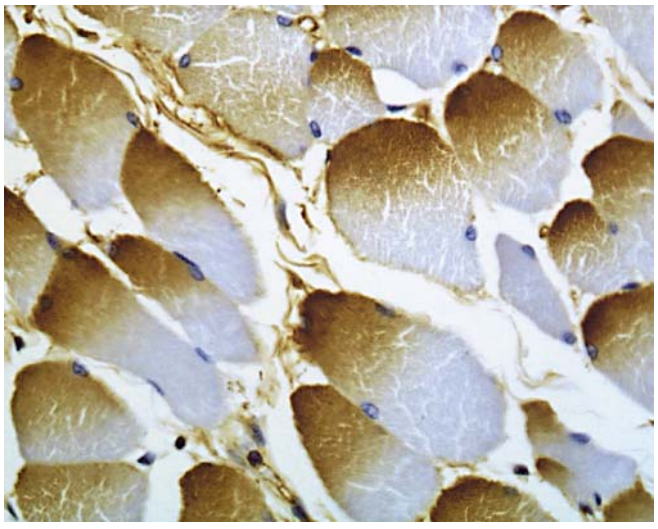

Fig. 4 Muscle fibers: intracellular reactivity for TN in a vital wound specimen (×400)

Table 3 Intracellular detection of FN and TN in skeletal muscle fibers

Group of rats (age of vital injury)	Vital	Postmortem
15 min.	2/8	3/7
10 min.	2/6	1/5
5 min.	3/6	1/4
Total	7/20	5/16

Discussion

Immunohistochemical methods have been widely investigated as possible tools in the diagnosis of vitality and to determine the age of cutaneous wounds. However, it is necessary to verify the validity of these techniques for those lesions produced at moments extremely near to death (before and after). This means establishing their ability to detect "low signal" vital reactions and to differentiate them from a supravital phenomenon occurring in the immediate postmortem period (Madea 1994). In real practice the determination between wounds that are caused at or immediately after death, i.e. within seconds, becomes a real problem for the forensic pathologist.

The investigations into FN and TN appear useful as they are glycoproteins of the extracellular matrix which play a key role in wound healing (Juhasz et al. 1993).

Immunohistochemical overexpression of FN has been described in short-evolution vital injuries of skin, and was found to be negative in postmortem wounds (Betz et al. 1992; Betz 1995). Otherwise, strong reactivity for TN has been observed in human injuries with a long time period of evolution, at least 1–3 days (Betz et al. 1993b; Betz 1995; Schenk et al. 1995).

In humans vital reaction of FN has been described by Betz et al. as the staining of the wound edge from which a network of irregularly ramifying fibers penetrate into the

immediately adjacent dermis (Betz et al. 1992; Betz 1995). This reaction was detected in wounds aged just a few minutes (Betz et al. 1992).

However, other authors have found differences both in the sensitivity of the technique and in the pattern of staining. With regard to sensitivity, we have previously reported this pattern of reaction in only 50% of a total of 38 vital wounds studied, where the reactive cases were aged at least 20 min, and with negative staining in all 4 samples aged just 3–10 min (Ortiz-Rey et al. 2002). In addition, differences concerning the pattern of staining have been reported in other species. Some authors have considered a positive reaction as a central wide reticular staining of the dermis not only adjacent to the margin, in porcine injuries (Grellner et al. 1998); in other series with mice, staining occurs mainly intracellularly in phagocytic macrophages of 2-day-old wounds (Kondo and Oshima 1996). On the other hand, the specificity of this reaction in an isolated study of postmortem wounds in pigs has been questioned (Grellner et al. 1998).

The present investigation on rat skin compared very short-aged vital and postmortem wounds that were inflicted in the same individuals. It has confirmed that the "network" staining pattern of FN, and also of TN, appears early, having occurred in most of the vital samples (90 and 80%, respectively). These findings are similar to those first reported (Betz et al. 1992) but are different to others (Ortiz-Rey et al. 2002). However, this pattern of staining was not absolutely specific and reactivity for FN and TN was found in some postmortem injuries (12.5 and 18.7%, respectively). Results reported for porcine samples show even less specificity with reactivity for FN in 50% of postmortem injuries incised in the very early postmortem period (Grellner et al. 1998).

The pattern of staining of FN and TN in our samples is somewhat different to that previously reported. Network reactions were not evident in the upper dermis but mainly in the subcutaneous tissue and adjacent skeletal muscle. This can be explained by the existence of larger blood vessels at this site compared to the dermis. This observation supports a passive mechanism for the expression of these substances in wounds. The rapid accumulation of FN in injuries, both in connective dermal tissue and in muscle cells, could be explained by the influx from damaged blood vessels, as has been previously suggested (Casscells et al. 1990; Fechner et al. 1993; Hu et al. 1996; Grellner et al. 1998). This mechanism could also explain the occasional postmortem positive staining seen in our present cases in rat and also found in pig wounds (Grellner et al. 1998).

The extremely high specificity reported in some human series could be questioned as it seems difficult to confirm negative results in postmortem specimens. Some of the studies have been made with samples obtained even 3 days after death (Betz et al. 1992; Betz 1995). Resistance of tissue antigens to autolysis varies for different molecules and thus has a decisive influence on immunohistochemical results (Fieguth et al. 1997). In fact, it has been reported that advanced autolysis and putrefaction induces negativity for FN (Ortiz-Rey et al. 2002) or background staining (Betz et al. 1993c) in human skin samples.

The absence of specificity is noteworthy with regard to skeletal muscle staining. Intracellular detection of FN after muscle trauma has been considered as a sign of vitality (Fechner et al. 1993). Vital reactions are described as an early accumulation of FN at the edges of the damaged fibres and subsequently as the reaction within the fibres, whereas postmortem injuries would not show these patterns of staining (Fechner et al. 1993). However, we have detected this reaction both in vital and postmortem wounds, with no statistical differences (35% vs. 31.25%, respectively). Staining of fiber cytoplasm has been partial. This particular pattern of reaction remains unexplained, but could result from technical problems.

Lastly, our investigation has shown a quite different immunoreactivity for TN in rat injuries compared to human skin. TN has been detected much earlier in rat than in human wounds, and a parallel reaction was observed with FN. This interspecies difference highlights, once again, the difficulties in applying the results from investigations on experimental animals to human practice, as has been pointed out (Knight 1996). Therefore, results from this investigation, performed in rats, and from other experiments in animals reported in the literature (Kondo and Oshima 1996;Grellner et al. 1998) can help to understand the problem but cannot be directly transferred to humans.

In conclusion, expression of FN and TN detectable through immunohistochemistry is more frequent in vital rat injuries. However, postmortem expression of these molecules occurred in a few cases. This seems to be related to the presence of these molecules in serum and their passive extravasation from damaged blood vessels, which could occur after death.

Although these results are not directly applicable to human species, they emphasize the need for caution when evaluating a wound. Natural features such as hemorrhages as well as technical problems (e.g. autolysis, putrefaction) can have a decisive influence on the results.

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