USE OF IMMUNOHISTOCHEMICAL EXAMINATION FOR DIAGNOSING AND DATING SKIN INJURIES IN FORENSIC MEDICINE IN SLOVAKIA

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Abstract: In forensic practice, defining the vitality of injuries and the time elapsed from the moment they occurred until the victim's death is one of the most difficult issues. Mechanical injuries are the most common injuries that a medical examiner encounters in everyday practice. However, the issue of the vitality and age of injuries is important even in the case of other types of violent deaths, e.g., in case of gunshot wounds, burns, hanging, strangulation, throttling, or drowning. Vital changes at the injury site can be assessed based on macroscopic, microscopic, and histochemical findings. Still, in recent years, the development of immunohistochemical techniques has made a significant contribution to more accurate diagnosis and dating of skin injuries. In forensic practice in Slovakia, these methods are not yet commonly performed, although they are an integral and necessary part of the forensic conclusions in foreign countries.

The aim of this study was to determine the presence and expression of selected antigens, namely fibronectin and tenascin, in injured skin and adjacent tissues, to distinguish injuries sustained during life from those which aroused after death, to specify the time of harm, and also to determine the possible effects of postmortem changes (autolysis, putrefaction) on the detection and relevance of the results. The acquired findings confirmed the importance of using immunohistochemical methods in dating skin and soft tissue injuries and their application to routine forensic practice in Slovakia, with significant use in expertise activities for law enforcement authorities.

Keywords: skin injuries, immunohistochemistry, fibronectin, tenascin, forensic medicine

INTRODUCTION

Defining the vitality of injury and the time that has elapsed from the moment of the injury to the victim's death is one of the most difficult issues in everyday forensic practice. The determination of the wound vitality and wound age estimation is based on the wound healing process. Skin and soft tissue excisions taken from the damaged areas at autopsy can be examined by conventional histological staining techniques. However, they do not always allow accurate dating of the injury. Histochemical evidence of enzymes is also crucial in assessing vital reaction and age of injury [1, 2]. Examination of human soft tissues using immunohistochemical methods is intended particularly for the distinctive differentiation of injuries that were incurred during life and those which aroused after death, as well as for distinction between fresh wounds and healing wounds with respect to postmortem changes occurring during the period from victim's death to the moment of the autopsy, and tissue collection for the additional laboratory expert examinations. The literature indicates some possibilities of using immunohistochemistry in the skin and soft tissue injuries. It is the detection of a number of immunohistochemical markers - glycophorin, fibronectin, laminin, tenascin, myoglobin, keratin 5, collagen types I, III, V, and VI, P-selectin, E-selectin, ICAM-1, VCAM-1, TGF-α, TGF- β, and others [2-7].

Based on the availability and possibilities of use in forensic workplaces, two immunohistochemical markers used in dating skin injuries – fibronectin and tenascin – were selected for our study.

Fibronectin is a high molecular weight

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glycoprotein found in the plasma and extracellular matrix of tissues. It is synthesized by a number of cells such as fibroblasts, endothelial cells, macrophages, hepatocytes, and astroglia. Human fibronectin has several structural domains (Hep1/Fib1, Gel, Cell, Hep2, Fib2) by which it binds to cell surfaces and molecules of collagen, heparin, gelatin, fibrin, and DNA. It plays an important role in mediating intercellular contact and, during healing, plays a role in cell adhesion, migration, hemostasis, phagocytosis, and angiogenesis [2, 8]. Research shows positivity of fibronectin in the vicinity of skin injuries already several minutes after wound infliction [1-7, 9].

Tenascin is a multifunctional high molecular weight glycoprotein also found in the extracellular matrix. It is synthesized by fibroblasts, osteocytes, chondroblasts, smooth muscle cells, and glial cells. Its occurrence is limited only to a healthy adult organism. Actively growing, migrating, and differentiating epithelial cells produce factors such as TGF-β that stimulate tenascin expression in the surrounding mesenchyme, be it tumors or physiological tissue repair [10]. Tenascin is detectable 2 to 3 days after skin injury [7].

Evidence of the presence of glycophorin and fibronectin at the injury site is essential in forensic medicine, as it makes it possible to distinguish between injuries incurred during life and agonal or postmortem wounds [6, 7]. In routine forensic practice, immunohistochemical methods themselves are practically non-utilized.

MATERIALS AND METHODS

In the presented study, a set of excisions from the skin and adjacent soft tissues was created, taken from deceased persons of various ages during autopsies at the Medico-legal Department of Health Care Surveillance Authority (HCSA). Excisions from the sites of multiple injuries were made, and two sets of excisions were created. The first group consisted of 15 surgical skin wounds with a known time of their occurrence. The collected excisions were harvested from the deceased with a survival time from 2 hours to 18 days after the operation. For practical reasons, it was impossible to collect surgical wounds with a short survival time of the individual, i.e., a few minutes to 2 hours. The second group consisted of 15 traumatic injuries, namely lacerations, skin abrasions, bruises, and burns with a known time of their occurrence. They were taken from the deceased with a survival time of several minutes to 5 days. The postmortem interval, i.e., the time between death and the autopsy with sampling, ranged from 8 to 96 hours. Tissue samples were fixed in 4% buffered formalin solution for 48 hours and embedded in paraffin. The prepared histological sections were stained using the conventional histological technique (hematoxylin-eosin staining) and the immunohistochemical method using the streptavidin-biotin complex. Mouse primary monoclonal antibodies against human fibronectin and tenascin (Sigma Aldrich) were used in the immunohistochemical method. Tissue sections were enzymatically digested with pepsin and proteinase K. Primary antibodies were diluted at a rate of 1:100 in PBS buffer. The tissue sections were incubated for 1 hour in a humid chamber at room temperature. An Ultra Vision LP detection system containing HRP Polymer (LabVision Corporation) was used. Diaminobenzidime, which was part of the detection system, was used to visualize the immunohistochemical reaction. Excisions from intact skins were used as a negative control. All slides were evaluated using a NIKON H550S light microscope at 100x, 200x, and 400x magnification.

RESULTS

In the case of positivity, both monitored antigens produced a net-like finding in the extracellular space, resembling strings, lumps, and nets, while these were present at the edges of the wounds. In the first set of surgical wound excisions, fibronectin was present at the edges of the surgical wounds in all excisions examined. In the second set, consisting of traumatic injuries and burns, the positivity of fibronectin was found in the extracellular matrix at the edges of the wounds about 10 minutes after its injury (Figs 1a, 1b, 2a, 2b). Tenascin focal positivity was observed in both sets after two days and diffuse positivity three days after the onset of skin damage (Figs 3a, 3b, 4a, 4b). The first set also served as a control to map the presence of antigens in the harvested tissues depending on the known time of surgery. Depending on the time of injury, various histopathological findings were found in hematoxylin-eosin staining, namely wounds with fresh hemorrhage, wounds with a leukocyte reaction of various degree, and repair processes up to scar-tissue wound healing. Detection of antigens in combination with hematoxylin-eosin staining has also made it possible to distinguish between injuries caused during a lifetime and injuries caused after death. One of the research aims was to determine the impact of postmortem changes on the detection and relevance of the results. It has been found
that the detection of fibronectin and tenasin is reliably possible within three days of an individual’s death. It is unusable in the case of late postmortem changes (putrefaction, mummification). When evaluating the results of immunohistochemical examinations, the effect of postmortem autolysis as an early postmortem change in terms of reduced immunoreaction, false positivity, or negativity was not observed in either

Figure 1. Laceration wound on the head (pedestrian traffic accident), survival approx. 10 minutes, fibronectin positivity.

Figure 2. Laceration wound on the head (ground-level fall), survival 30 minutes, fibronectin positivity.

Figure 3. Surgical wound on the head (craniectomy), survival 2 days, tenasin positivity.
case. At the same time, no differences in the detection and expression of fibronectin and tenascin were found depending on the age and individual disease changes.

**DISCUSSION**

In their study, Liu, Chen, and Huang examined fibronectin expression in rat skin contusion samples immunohistochemically using the EIIIA monoclonal antibody. All examined skin excisions manifested positive staining. The intensity of the staining was dependent on the time of the injury, increasing in correlation with the age of the wound. Healthy skin did not show positive staining [11]. Takamiya et al. in their study found fibronectin mRNA expression in injured mice's skin. Fibronectin mRNA expression peaked eight hours after injury, reaching baseline expression after 24 hours [12].

Immunohistochemical detection of fibronectin appears to be effective in determining the age of injuries in the early post-traumatic interval. Balažić et al. found positive fibronectin expression in gunshot wound samples in cases with a survival time of 10-20 minutes. In cases where injuries were caused just before death, the samples were similar to staining in postmortem samples [13]. Dettmayer reports that fibronectin is present as early as several minutes after injury [7]. Fieguth et al. investigated fibronectin expression of other markers in human surgical wounds taken from individuals who died immediately following fatal trauma (aircraft accidents, train collisions). They found that immunohistochemical detection of fibronectin in fresh wounds 20-30 minutes after a trauma is possible [14]. Grellner et al. in a study on pig skin found that even a postmortem injury could lead to fibronectin positivity [15]. Betz et al. followed surgically treated skin wounds in persons with a survival of 8 hours to 7 months after surgery. Tenascin was visualized immunohistochemically pericellularly around fibroblasts two days after injury at the earliest. Net-like staining positivity was detected in the 3-day-old wound; in all wounds older than 5 days, intense tenascin reactivity at the wound sites (dermal-epidermal junction, wound edge, bleeding site) was observed. In wounds older than 1.5 months, staining was not positive at the scar site [16]. Toupalik states that the occurrence of intensely responsive structures resembling strings, which form the basis of the fibronectin net at the edges of wounds and in hemorrhages, can be considered a positive reaction of fibronectin [17].

The results of our work coincide with the results of previous foreign studies. The use of immunohistochemical methods is still the subject of research using the search for more suitable antibodies against substances or structures of various tissues and organs in various causes of death. In the case of defining the vitality and dating of skin injuries, the selection of suitable markers is crucial, both in terms of the estimated time of injury but also in line with the available instrumentation, material, and technological equipment of a particular workplace. The larger the number of markers evaluated at the same time, the more accurate the ability of injury dating [18, 19]. In forensic medicine, immunohistochemistry is used worldwide for brain trauma, early ischemic myocardial changes (myoglobin, desmin) [20-22], sudden infant death syndrome, signs of drowning (presence of AQP-4 receptors in the brain) or hypothermia (MAP-2 in the hippocampus, or HSP70 in the kidneys) and the like [23, 24]. Immunohistochemical methods
are practically unused in routine forensic practice in Slovakia, although they are an integral and necessary part of forensic assessment abroad.

**In conclusion**, when used correctly and appropriately, the combination of conventional histology and immunohistochemical examination (detection of selected antigens) in injured skin and adjacent tissues makes it possible to specify the time of injury and, at the same time, determine the vitality of injuries more accurately. The findings acquired from this work confirmed the importance of the introduction and justification of the use of immunohistochemical methods in dating skin and soft tissue injuries. The results obtained can be applied in daily forensic practice in Slovakia. Utilizing immunohistochemical methods together with a complete external examination of the body, thoroughly performed autopsy, and laboratory findings would contribute to answering a plethora of medico-legal questions.

**Conflict of interest**
The authors declare that they have no conflict of interest.

**References**