Immunohistochemistry as a tool to characterize human skin wounds of hanging marks

I. Legaz1,*, M.D. Pérez-Cárceles1, M. Gimenez2, F. Martínez-Díaz1, E. Osuna1, A. Luna1

Abstract: Estimation of age and vitality of human skin wounds both in the living and dead is essential in forensic practice. The use of immunohistochemical parameters for the age estimation and vitality of human skin wounds remains difficult. Forensic literature describes different biomarkers and methods for the differential diagnosis of vital and post-mortem wounds, but to this day it is unclear its utility. The aim of this study was analysed wounds vital origin in a series of suicidal hangings were vitality had been demonstrated using fibronectin, cathepsin D and P-selectin, in order to discover the morphological changes that occur in a vital wound, and consequently, find useful vital injury diagnosis markers. A total of 15 human vital skin wounds from ligature marks from deaths by suicidal hanging and skin controls from same cadaver were analyzed in a postmortem interval between 19-36 hours. Fibronectine, cathepsin D and P-selectin were detected by immunohistochemistry. Our result shows a strongly fibronectin-positive reaction in basement membranes and interstitial connective tissue in all specimen of wounds of ligature mark. Granular staining pattern characteristic of cathepsin D was observed mainly in the basal layer of the epidermis in normal and wound skin. Cathepsin D analysis in ligature mark showed moderate positive and strong positive cells. A weak positive immunoreactivity P-selectin were found in vital wound compared with undamaged skin. In conclusion, our data show an increase of fibronectin and cathepsin D immunoreactivity expression and a decrease of P-selectin immunoreactivity in skin wounds from ligature marks from deaths by suicidal hanging of a postmortem interval between 19-36 hours.

Key Words: cathepsin D, fibronectin, P-selectin, skin wounds, suicidal hanging.

INTRODUCTION

Estimation of age and vitality of human skin wounds both in the living and dead is essential in forensic practice. The use of immunohistochemical parameters for the age estimation and vitality of human skin wounds remains difficult, due to supravital reactions and the minimal morphological changes present during this time [1–4].

Classically, it has been considered that extravasations of red blood cells and haemoglobin to the wound was a vital reaction sign, but several studies contradict these observations, concluding that cannot be used as a reliable marker in wound vitality diagnosis [5]. Hanging deaths are common and characterized by a very brief time of survival. The death usually occurring within minutes or over the first 24 hours. If the person survives the initial event, later he/she may die because of the severity of the initial hypoxic and ischemic brain damage (Langlois and Gresham 1991). In certain circumstances it is necessary to discriminate antemortem wounds from postmortem damage in human cadavers [5]. Often macroscopic and histological exams are unable to distinguish vital lesions from post-mortem lesions.

1) University of Murcia, Faculty of Medicine, Regional Campus of International Excellence “Campus Mare Nostrum”, Biomedical Research Institute (IMIB), Department of Legal and Forensic Medicine, Murcia, Spain
* Corresponding author: University of Murcia, Faculty of Medicine, Regional Campus of International Excellence “Campus Mare Nostrum”, Biomedical Research Institute (IMIB), Department of Legal and Forensic Medicine, E-30110, Murcia, Spain, Tel: (+34) 868883957, Fax: (+34) 868834307, E-mail: isalegaz@um.es
2) Institute for Legal Medicine, Sao Paulo, Brazil
There are many immunohistochemical markers used to characterize wounds [1, 6, 7]. Selectins are adhesion molecules that can help to improve wound age estimation, especially in injuries with a short survival time, this also being indicative of wounds vitality [8, 9]. Selectins are carbohydrate-binding molecules that bind to fucosylated and sialylated glycoprotein ligands [10], and in the case of P-selectin (CD62P) have been studied for vital and postmortem wounds differential diagnosis [8, 9].

The enzymatic markers Cathepsin D plays an important role in certain apoptotic processes, protein turnover and protein processing [11, 12] and as wound vitality markers have been extensively studied [13], but there is not clear response on its potential usefulness [14–16], because it has been found at high expression level in vital and post-mortem injuries [16]. On the other hand, fibronectin, a 440 kDa glycoprotein, is a component of basement membranes and interstitial connective tissue [17] and circulates in the plasma. Fibronectin plays an important role in cell adhesion and cell migration during wound healing [17], and also involved in angiogenesis during wound healing and in wound contraction [18].

However, use of specific vitality biomarkers with an appropriate and standardized methodology is still matter of debate to clarify the relationship between wounds and death cause [4, 19]. The aim of this study was to characterize human skin wounds of hanging marks using fibronectin, cathepsin D and P-selectin to establish wounds vitality in a series of suicidal hangings were vitality had been previously demonstrated.

**MATERIAL AND METHODS**

Human skin specimens

A total of 15 human vital skin injuries from ligature marks from deaths by suicidal hanging and undamaged skin from same zone and cadaver were analyzed. In both cases, skin specimen corresponded to skin cross sections of 0.5-0.7 cm. None of the cases had suffered from severe malnutrition, malignant diseases or metabolic disorders, and no substances such us cytostatic agents or glucocorticoids, which may possibly influence wound healing, were administered during medical treatment. In all cases people were found dead and skin specimens were collected at autopsy within a post-mortem interval of between 19 and 36 hours from the discovery of the body. Permission for this study was given by the relevant institutional ethical committee.

**Immunohistochemistry**

Two skin specimens were used to compare different immunomarkers, a ligature mark zone specimen (n=15) and a control skin specimen from same individual (n=15), both corresponded to skin cross sections with similar dimensions (0.5-0.7 cm).

To detect the protein expression of cathepsin D and P-selectin in human skin wounds, specific immunohistochemistry was used. Specimens were fixed overnight by immersion in phosphate-buffered 4% paraformaldehyde (pH 7.4) at 4°C. After extensive washing in 0.1M phosphate-buffered saline (PBS; pH 7.4), the specimens were embedded in paraffin for sectioning into transversal skin sections using a microtome (Leica RM2255, Germany; 60 μm thick). To analyze the cytoarchitecture, sections were stained with haematoxylin-eosin and also were processed for immunohistochemistry to detect fibronectin, cathepsin D and P-selectin. For immunohistochemical study, sections were deparaffinized and incubated at 37°C for 24 hours, after blocking endogenous peroxidase with 3 % H₂O₂ in PBS-T (Tween 20) for 15 minutes. Sections were subsequently incubated 30 min at 25°C, under constant shaking with the following primary antibodies: monoclonal mouse anti-human cathepsin D (clone DB2000, Dako; diluted 1:400), monoclonal mouse P-selectin (clone 1E3, Dako; diluted 1:100), and polyclonal rabbit fibronectin (Dako, diluted 1:5000, Dako). After extensive washes in PBS-T, sections were incubated in a secondary biotinylated goat anti–mouse IgG (Vector, Burlingame, CA; diluted 1:200) for 1 hour, washed again in PBS-T, and incubated in streptavidin–biotin complex (ABC Kit, Vector; 0.003% dilution) for 1 hour. Immunolabeling was revealed with 0.05% diaminobenzidine (DAB; Sigma) in 0.05 M Tris (pH 7.6), containing 0.03 % H₂O₂. As a control of the immunohistochemical method used in the present study, sections were processed as indicated but the corresponding primary antiserum was replaced by rabbit or mouse no immune serum (1:500). No immunostaining could be detected under these conditions.

Finally, digital photographs were taken on a Zeiss Microscope equipped with a Zeiss digital camera. Digital images were adjusted for brightness/contrast by using Adobe Photoshop (San Jose, CA); no additional filtering or manipulation of the images was performed. Figures were mounted and labelled by using Power Point 2016 (Microsoft corporation, Seattle, WA).

**RESULTS**

**Characterization of the skin wound**

The samples of skin wounds come from deaths by suicidal hangings autopsied corresponding to men (80%, n=12) with a mean age of 34.2 ± 12.3 (years ± SD) and a mean body weight of 70.7 ± 5.6 Kg. Women were only represented in a 20% (n=3) with a mean age of 33.0 ± 12.3 years ± SD) with mean body weight of 56.2 ± 9.0 (Kg ± SD). Most of the neck injuries that occurred affected only subcutaneous structures, not muscle or bone structures.

**Fibronectin in human skin wounds of hanging marks**

In normal undamaged skin, fibronectin showed weak positive reaction in the basement membrane zone...
of the dermal-epidermal junction and vasculature in the skin. Weak immunostaining was observed in fibronectin strands in the loose connective tissue (Fig.1A). Conversely, strongly fibronectin-positive reactions could be observed in basement membranes and interstitial connective tissue in all specimen of wounds of ligature mark included in our study (Fig.1B).

**Cathepsin D and P-selectine in human skin wounds of hanging marks**

Different expressions were found to cathepsin D between control skin and ligature mark. Granular staining pattern characteristic of cathepsin D was seen in 98% cases, which was focally expressed mainly in the basal layer of the epidermis in normal and wound skin. In normal skin, the intensity of staining ranged between mild degree to moderate in all cases (Fig. 1C), but nevertheless an increase in cathepsin D expressions were observed in skin wounds of hanging marks. Cathepsin D analysis in ligature mark showed moderate positive and strong positive cells (Fig. 1D). On the other hand, a differential P-selectin expressions were observed by comparing control skin with ligature mark. A moderate positive immunoreactivity P-selectin were found in vital wound compared with normal skin (Figs 1E, F).

**DISCUSSION**

This study analysed wounds vital origin in a series of suicidal hangings were vitality had been demonstrated using fibronectin, cathepsin D and P-selectin, in order to discover the morphological changes that occur in a vital wound after death, and consequently, find useful vital injury diagnosis markers.

Demonstrate the vitality of a wound in the post-traumatic interval is difficult, due to the supravital reactions and the morphological changes that occur during this time, and for this reason there are many methods and biomarkers that are being studied [20]–[22].

Fibronectin is high - molecular - weight glycoprotein that plays a crucial role the wound healing process and tissue repair, particularly in extracellular matrix formation and also in reepithelialisation, cell adhesion, migration, metastasis, proliferation and differentiation [23, 24]. This glycoprotein have been studied for estimating the age of skeletal muscle damage [25], ischemic myocardial lesions [26, 27], and their distribution in human skin wounds [28, 29], such us their expression in cell carcinoma and its role in metastasis [23]. Our results on vital wound within a post-mortem interval of between 19 and 36 hours show strongly fibronectin-positive reactions in basement membranes and interstitial connective tissue. Similar results were obtained in other studies where massive networks of fibronectin were detected in wounds at least after 40 min-

old and also strongly positive fibronectin complexes were detected in the oldest wounds examined (13 and 30 days) [30]. On the contrary, previous literature reports that fibronectin could be a marker of vitality for wounds with a survival time of more than a few minutes [31]. Other studies also detected a marked fibronectin reaction using wounds induced in narcotised pigs in endotoxic shock in the very early post-mortem period [31]. Ortiz-Rey et al. 2002, observed a reticular staining for fibronectin only in vital specimens but not in post-mortem cases. Other authors, evidenced that fibronectin is a useful marker for vital wounds with a survival time of more than a few minutes [29]. Curiously, a study about chronic diabetic foot wounds observed that a new negative-pressure wound therapy can putatively facilitate wound healing since it increases the expression of fibronectin and TGF-β1 in diabetic foot wounds avoiding amputation, morbidity, and hospitalization for patients with diabetes [23].

On the other hand, our result about cathepsin D show a high positive cathepsin D expression in ligature mark respect to control skin according to the previous study [16]. Similar studies, carried out in pig and human skin at different times showed cathepsin D was a useful marker for differential vital and postmortem wounds [32, 33]. On the contrary, other study excludes any usefulness

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**Figure 1.** Transversal skin sections stained with fibronectin, cathepsin D, P-selectin and haematoxylin-eosin in undamaged skin (A, C, E) and ligature mark (B, D, F). Brown cells show fibronectin, cathepsin D or P-selectin positive cells and blue cell show tissue architecture stained with haematoxylin-eosin (HE). Black arrowheads show the cells marked in each immunohistochemistry, respectively. A, B and F, with 10x magnification and C, D and E with 20x magnification.
of immunohistochemistry quantification of this enzyme in the differentiation between vital and post-mortem injuries [16]. A studied realized in brain injury after subarachnoid haemorrhage suggest that the lysosomal membrane may be damaged after subarachnoid haemorrhage, which leads to the release of proteases (cathepsin B/D) and activates the apoptotic pathway. In this study, levels of cathepsin B/D were up-regulated and peaked at 48-h post-subarachnoid haemorrhage [34].

The analysis of P-selectine show a moderate positive immunoreactivity expression in vital wound respect to control skin corroborating previous studies where P-selectin expression intensity was increased up to a wound age of 1 h, and after that a decrease in staining intensity was evidenced, such that older skin wounds clearly show a decrease in staining intensity [35]. Other authors, observed an elevated expression of P-selectin expression in injuries occurred during live [36], but nevertheless, other studies confirmed that P-selectin was detected in post-mortem lesions and concluded that it is not specific to vital injuries [37]. A limitation of our study could be that our ligature mark samples were not collected at the same death time, which may influence fibronectine, P-selectin and cathepsin D immunoreactivity observed in our data, because its expression is time related.

In conclusion, our data show an increase of fibronectin and cathepsin D immunoreactivity expression and a decrease of P-selectin immunoreactivity in skin wounds from ligature marks from deaths by suicidal hanging of a postmortem interval between 19-36 hours.

**Conflict of interest.** The authors declare that there is no conflict of interest.

References