Histol Histopathol (2010) 25: 1539-1546

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Tryptase, CD15 and IL-15 as reliable markers for the determination of soft and hard ligature marks vitality

Emanuela Turillazzi[1], Giuseppe Vacchiano[2], Aurelio Luna-Maldonado[3], Margherita Neri[1], Cristoforo Pomara[1], Roberto Rabozzi[1], Irene Riezzo[1] and Vittorio Fineschi[1]

[1]Department of Forensic Pathology, University of Foggia, Ospedale Colonnello D'Avanzo, Foggia, Italy, [2]Institute of Legal Medicine, University of Sannio, Benevento, Italy and [3]Legal Medicine Area, University of Murcia, Espinardo, Murcia, Spain

Summary. In forensic practice, it is required to distinguish between suicidal or accidental hanging and simulated hanging. Conventional macroscopic and histological findings may be unreliable; vital signs are often absent, and they can be produced postmortem. The application of immunohistochemical techniques opened up a new field of investigation in the issue of ligature marks. We investigated the immunohistochemical expression of a panel of cytokines and inflammatory cells in skin specimens in autopsy cases of death due to hanging, to discuss their significance in assessing whether hanging mark and signs occurred before or after the death of the victim. We selected 21 cases in which broad, soft and yielding materials were used and 28 cases in which materials used for hanging were hard. The control group included the following 21 cases: 14 cases of sudden cardiac death and 7 cases of post-mortem hanging (suspension) of bodies (drug overdose or suffocation as cause of death in all the cases). An immunohistochemical investigation of skin samples was performed utilizing antibodies anti-tryptase, fibronectin, TNFα, IL-6, IL-8, IL-10, MCP-1, IL-15, IL-1ß, CD45, CD4, CD3, CD8, CD68, CD20, CD15. We conclude that tryptase, IL-15, and CD15 appear to be reliable parameters in the determination of ligature marks' vitality with the accuracy needed for forensic purposes. This fact especially applies to soft marks which are particularly difficult to evaluate on the basis of gross examination and of conventional histological studies.

Offprint requests to: Prof. Vittorio Fineschi, Department of Forensic Pathology, University of Foggia, Ospedale Colonnello D'Avanzo, Viale degli Aviatori 1, 71100 Foggia, Italy. e-mail: vfinesc@tin.it

Key words: Hanging, Ligature marks, Vitality, Tryptase, IL-15, CD15

Introduction

Hanging is a common suicidal option; hanging as a cover – up for murder by some other means is rare, but it presents special problems for forensic pathologists (Leth and Vesterby, 1997). Homicide with subsequent hanging to create the impression of suicide can remain undiscovered or cannot be excluded, so thorough gross examination and careful histological investigation are mandatory (Janssen, 1984). Ligature marks (patterned abrasion caused by ligature material) can be of great significance to the forensic pathologist in determining the cause and manner of death. Indeed, the gross examination of these marks may, sometimes, be unreliable and may mislead the forensic pathologist in drawing conclusions as to whether it is due to hanging or post – mortem suspension of the body. Many difficulties still exist, particularly when broad, soft and yielding materials are used for hanging and the mark is discontinuous or even absent, and if the body was suspended for short time, insufficient for reactions to develop and become reliably distinguished from lesions of post mortem origin (Janssen, 1984). Extreme care should be taken in describing and interpreting the marks in such cases before giving an opinion regarding the manner of death. Immunohistochemistry studies may lead to reliable information for a forensic ligature marks vitality estimation. Previous studies have substantially established that neutrophils that have migrated to an inflammatory focus exhibit an altered ability (usually upregulated) to express/produce chemokine(s),

supporting the idea that neutrophil-derived chemokines may contribute to the regulation of leukocyte accumulation (Scapini et al., 2000).

In this study we investigated the immunohistochemical expression of a panel of cytokines and inflammatory cells in skin specimens in autopsy cases of death due to hanging, to clarify and to discuss their significance in assessing whether hanging mark and signs occurred before or after the death of the victim.

Material and methods

We retrospectively analyzed the autopsy records of the Department of Forensic Pathology, University of Foggia (Italy) and Institutes of Legal Medicine of the University of Sannio (Italy) and Murcia (Spain) between 2001 and 2008. As for analysis of death scene investigation and autopsy reports, together with the information gathered from the police, 49 cases of hanging fatalities of suicidal origin were selected. Cases with poor or deficient information about the manner of death were excluded. Decomposed bodies were excluded from the study too. As for the type of hanging material used for ligature, we selected 21 cases in which broad, soft and yielding materials were used and 28 cases in which materials used for hanging were hard. The control group included the following 21 cases: 14 cases of sudden cardiac death and 7 cases of post-mortem hanging (suspension) of bodies (drug overdose or suffocation as cause of death in all cases). The deceased were 40 men and 30 women, ranging in age from 20 up to 50 years (average 29.16 years).

In all cases of hanging, sections of skin were removed from the neck at the site of the greater depth of the marks. In control cases skin samples were taken from the anterior face of the neck.

A routine microscopic histopathological study was performed using haematoxylin-eosin (H&E) staining. In addition, immunohistochemical investigation of skin samples was performed utilizing antibodies anti-tryptase, fibronectin, TNFα, IL-6, IL-8, IL-10, MCP-1, IL-15, IL-1ß, CD 45, CD 4, CD 3, CD 8, CD 68, CD 20, CD 15.

We used 4 μm thick paraffin embedded sections, mounted on slides covered with 3, amminopropyl-triethoxysilane (Fluka, Buchs, Switzerland). The sections in paraffin were re-hydrated and incubated for 20 minutes in methanol containing 10% of H_2O_2 to block endogenous peroxidases. The sections were pretreated to facilitate antigen retrieval and to increase membrane permeability to antibodies and then incubated with the primary antibody (see Table 1). The detection system utilized was the LSAB+ kit (Dako, Copenhagen, Denmark), a refined avidin–biotin technique in which a biotinylated secondary antibody reacts with several peroxidise conjugated streptavidin molecules. The positive reaction was visualized by 3,3-diaminobenzidine (DAB) peroxidation, according to standard methods. The sections were counterstained with Mayer's haematoxylin, dehydrated, coverslipped and observed in a Leica DM4000B optical microscope (Leica, Cambridge, UK) connected to a computerized system with photo camera (DC 480 Leica, Cambridge, UK).

A semi-quantitative evaluation of the immunohistochemical findings by two different investigators (MN, IR) without prior knowledge was performed; all measurements were done at the same magnification of image (x10) and the following gradation of the immunohistochemical reaction was used in the scale 0-4, as follows:

- (0): not expressed,
- (+): isolated and disseminated expression,
- (++): expression in groups or widespread foci,
- (+++): widespread expression,
- (++++): massive and diffuse positivity.

The samples were also examined under a confocal

Table 1. Panel of antibodies investigated.

Antibody against	Pre-treatment	Incubation time of primary antibody, temperature	Concentration of primary antibody
Fibronectin (DAKO, Copenhagen, Denmark)	Proteinase K (T: 20°C per 15 min)	120 min, 20°C	1:300
Tryptase (DAKO, Copenhagen, Denmark)	5 min Proteolytic Enzyme (Dako, Copenhagen, Denmark), 20° C	120 min, 20°C	1:1000
TNFα (Santa Cruz, CA, USA)	boiling in 0.1 M Citric Acid buffer.	120 min, 20°C	1:600
IL-6 (Santa Cruz, CA, USA)	5 min Proteolytic Enzyme (Dako, Copenhagen, Denmark), 20°C.	120 min, 20°C	1:2000
IL-8 (Abcam, Cambridge, UK)	5 min Proteolytic Enzyme (Dako, Copenhagen, Denmark), 20°C.	120 min, 20°C	1:500
IL-10 (Peprotec, London, UK)	5 min Proteolytic Enzyme (Dako, Copenhagen, Denmark), 20°C.	120 min, 20°C	1:4000
IL-15 (R&D Systems, Inc. Minneapolis, USA)	boiling in 0.25 mM EDTA buffer.	120 min, 20°C	1:100
IL-1, (Santa Cruz, CA, USA)	boiling in 0.25 mM EDTA buffer.	120 min, 20°C	1:4000
CD 45 (DAKO, Copenhagen, Denmark)	boiling in 0.25 mM EDTA buffer.	120 min, 20°C	1:600
CD 3 (DAKO, Copenhagen, Denmark)	boiling in 0.25 mM EDTA buffer.	120 min, 20°C	1:100
CD 4 (Santa Cruz, USA)	boiling in 0.25 mM EDTA buffer.	120 min, 20°C	1:50
CD 8 (DAKO, Copenhagen, Denmark)	boiling in 0.25 mM EDTA buffer.	120 min, 20°C	1:100
CD 20 (DAKO, Copenhagen, Denmark)	boiling in 0.25 mM EDTA buffer.	120 min, 20°C	1:200
CD 68 (Serotec, United Kingdom)	5 min Proteolytic Enzyme (Dako, Copenhagen, Denmark), 20°C.	120 min, 20°C	1:200
CD 15 (DAKO, Copenhagen, Denmark)	boiling in 0.25 mM EDTA buffer.	120 min, 20°C	1:50

microscope and a three-dimensional reconstruction was performed (True Confocal Scanner, Leica TCS SPE).

Statistical analysis

Semi-quantitative evaluation of the immunohistochemical findings and gradation of the immunohistochemical reaction were described with an ordinal scale and the median value reported.

Analysis of variance for the non parametric data was performed using Kruskal-Wallis test. When differences were found to be significant, analysis between the unmatched groups were elucidated with a Dunn's Multiple Comparison post hoc test. Significance level was set to 5% (SPSS ver. 16.01 for Windows - SPSS Inc., Chicago USA).

Results

External examination of the bodies gave no significant findings. Frequent findings at autopsy included pulmonary oedema, congestion, and focal pulmonary atelectasis. The microscopic observation of the skin specimens from hanging marks presented formation of intra-epidermal liquid-filled vesicles, in a few cases dermal mild leukocytes reactions and alteration of the musculature in the form of Zenker's necrosis.

In the cases of post-mortem suspension of bodies, plethora of the dermal vessels and metachromasia of the dermal and sub-dermal connective tissue were observed.

Immunohistochemistry showed a patchy dermal strong positivity of CD15, tryptase, and IL-15 reaction in

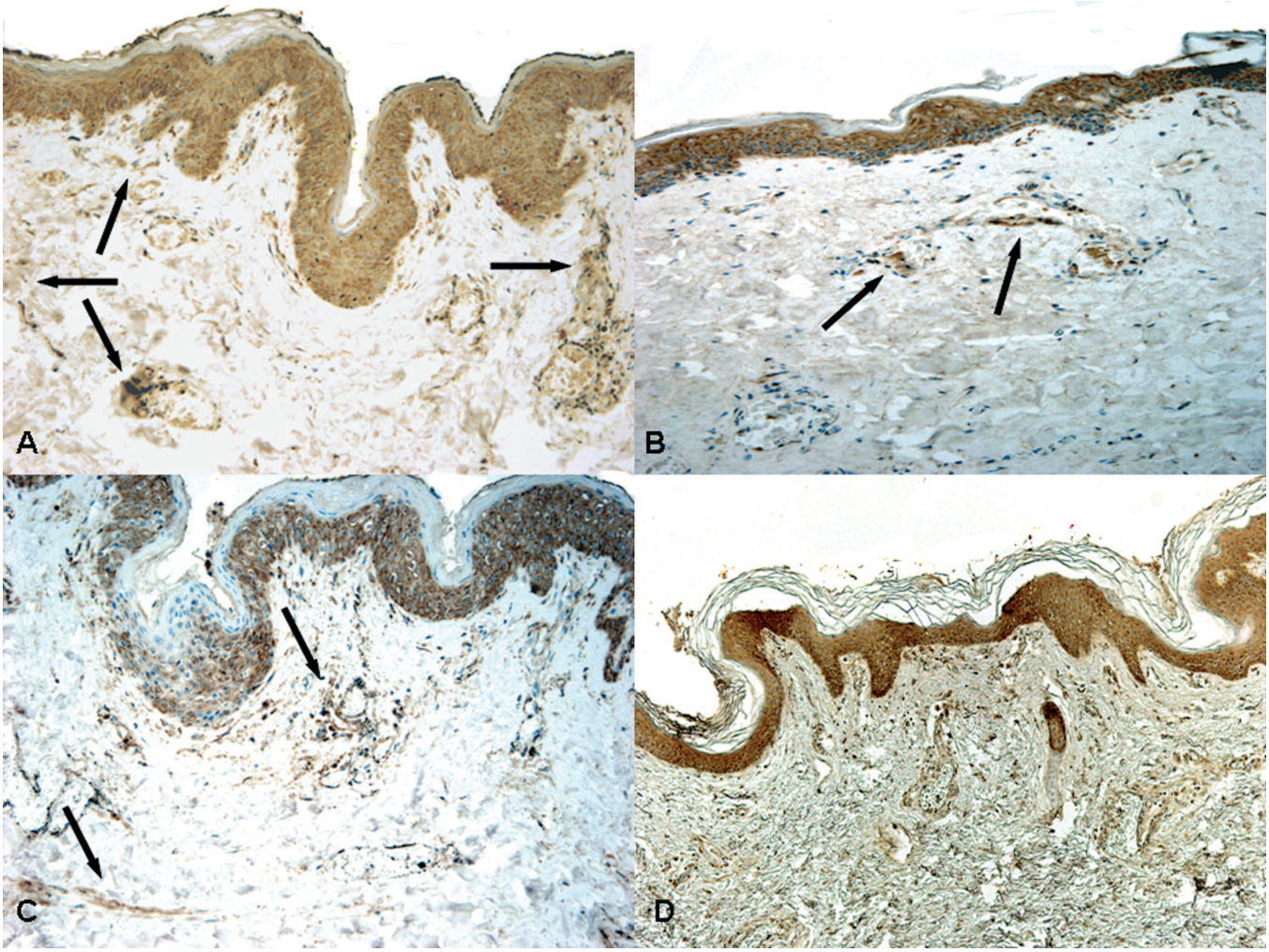

Fig. 1. The immunodetection of IL-15 was restricted to microfocal sites and was typical of most perivasal spaces (**A-C**), it was associated with a diffuse reaction in a minority of hanging specimens (**D**). A, x 60; B, D, x 20; C, x 40

Table 2. Semi-quantitative evaluation and statistical analysis of the immunohistochemical findings and gradation of the immunohistochemical reaction in the marginal zones above and below the hanging marks.

Antibody	A Soft marks 21 cases	B Hard marks 28 cases	C Controls 21 cases		Statistical value
Fibronectin	-	-	-		NS
				A vs B	NS
				A vs C	NS
				B vs C	NS
Tryptase	++++	++++	-		***
				A vs B	NS
				A vs C	***
				B vs C	***
IL-6	+	+	-		***
				A vs B	NS
				A vs C	***
				B vs C	***
IL-1ß	-	-	-		NS
				A vs B	NS
				A vs C	NS
				B vs C	NS
IL-8	+	+	-		***
				A vs B	NS
				A vs C	*
				B vs C	***
IL-10	-	-	-		NS
				A vs B	NS
				A vs C	NS
				B vs C	NS
IL-15	++++	++++	++++		***
				A vs B	NS
				A vs C	***
				B vs C	***
TNF-α	-	-	-		NS
				A vs B	NS
				A vs C	NS
				B vs C	NS
CD45 (leukocyte common antigen)	+++	+++	+++		***
				A vs B	NS
				A vs C	***
				B vs C	***
CD3 (T cell receptor complex)	+	+	-		***
				A vs B	NS
				A vs C	***
				B vs C	***
CD4 (T helper cells)	+	+	-		***
				A vs B	NS
				A vs C	***
				B vs C	***
CD8 (cytotoxic T cells)	+	+	-	***	
				A vs B	NS
				A vs C	***
				B vs C	***
CD15 (neutrophils)	++++	++++	-		***
				A vs B	NS
				A vs C	***
				B vs C	***
CD20 (B-lymphocyte antigen)	+	+	-	***	
				A vs B	NS
				A vs C	***
				B vs C	***
CD68 (macrophages)	+	+	-		***
				A vs B	NS
				A vs C	***
				B vs C	***

NS: p>0.05; *: p<0.05; **: p<0.01; ***: p<0.001. Intensity of immunopositive infiltrates were assessed semiquantitatively in the scale 0–4 as follows: -: no immunoreactivity (0%); +: mild immunopositivity in scattered cells (10%); ++: immunopositivity in up to a third of cells (33%); +++: immunopositivity in up to a half of cells (50%) and ++++: strong immunopositivity in the majority or all cells (100%).

the marginal zones above and below the hanging marks (Table 2). The microscopic observation of the samples showed the following structural differences: IL-15 was located around the dermal vessels and diffusely sparse in sub-dermal connective (Fig. 1); CD15 (Fig. 2) and tryptase (Fig. 3) reactions were intense in dermal connective tissue. When CD15 reaction was present, IL-15 positivity was always observed to denote the earlier reaction referring to CD15 (Fig. 4), acting as a pro-inflammatory cytokine.

In postmortem injuries and in uninjured skin specimens no immunohistochemical positivity was found which could be confused with positive reactions observed in vital ligature marks. The histological examination of control skin tissues was unremarkable too.

Discussion

To the best of our knowledge, this study is the only one focusing on the application of immunohistochemistry in assessing hanging marks'vitality based on cytokine response. We investigated a panel of cytokines, multifunctional glycoproteins, which are closely involved in various biological events, including the local inflammation response. As has been pointed out (García-Ramallo et al., 2002), of the myriad of mechanisms that support the host's initial defence system, none is more important than the successful recruitment of leukocytes from the lumen of a vessel to an area of inflammation. This seemingly simple event is supported by a sophisticated process of cytokine cascades, cell-to-cell communication, and the expression of redundant chemotactic mediators that collectively aid in the delivery of specific leukocyte subpopulations to a restricted area of tissue injury. Data from the set of studies demonstrated that resident tissue cells are a significant component of the initial localized in vivo response, a reaction that is dictated by the sequence of cytokine-chemokine expression (García-Ramallo et al., 2002). We immunohistochemically detected a strong IL-15 expression in skin specimens of hanging cases. IL-15 is a cytokine identified on the basis of biological activities similar to IL-2, and IL-15 is reportedly expressed by activated monocytes, epithelial cells and fibroblasts. A previous study showed that IL-15, in addition to its effect on T cells, also exhibits effects on monocytes, and may act as a proinflammatory cytokine, inducing monocytes to secrete both neutrophils and monocyte chemotactic factors (Badolato et al., 1997). Our choice of IL-15 is explained by the synergism with neutrophilic granulocytes and our study shows the potential for striking cytokine synergy in promoting fast, local neutrophil response in damaged tissues. Neutrophils are major players in inflammation and are known to express all components of the IL-15. IL-15 can induce phagocytosis, cytoskeleton rearrangement, gene expression, *de novo* protein synthesis and can delay apoptosis in human neutrophils. Production of

chemokines, cytokines and natural inhibitors is increased in IL-15-induced neutrophils, including CXCL8 (IL-8), IL-1ß, sIL-1RII and IL-1Ra (Pelletier and Girard, 2005). However, it is well known that cells which usually occur in blood (e.g. neutrophilic and eosinophilic granulocytes, macrophages without phagocytosed material or lymphocytes) are sometimes found in considerable amounts in the areas of bleeding of postmortem wounds (Betz, 1994).

In hanging cases, peripheral blood neutrophils are the first responder cells to arrive in significant numbers during the evolution of an acute, local inflammatory response. Positive reactions, defined as the presence of more than 10 cells outside the areas of bleeding, were first detectable in skin wounds aged about 20-30 min (Betz, 1994; Kim et al., 2008). In experimental studies on leukocyte recruitment during local inflammation, neutrophils were the predominant cell type at all time points (0, 2, 4, 6, 14, 24, 48, and 72 h) after the injection of an inflammatory stimulus (García-Ramallo et al., 2002). As expected, CD15 is an adequate parameter in detecting the local cutaneous and subcutaneous neutrophils response to the trauma due to hanging, even if no evident signs of compression of the soft parts of the neck are present.

In addition to neutrophilic granulocytes recruitment, we investigated mast cell activation in the site of blunt trauma due to pressure and abrasion by ligature materials. Because mast cells contain a variety of potent mediators, including histamine, heparin, proteinases, leukotrienes and multifunctional cytokines, their potential contributions to the processes of inflammation have recently become evident. Histamine levels in skin vary appreciably after wounding, and the levels increase significantly between 5 min and 3 h after trauma and decrease afterwards until 24hs (Betz, 1995). Furthermore

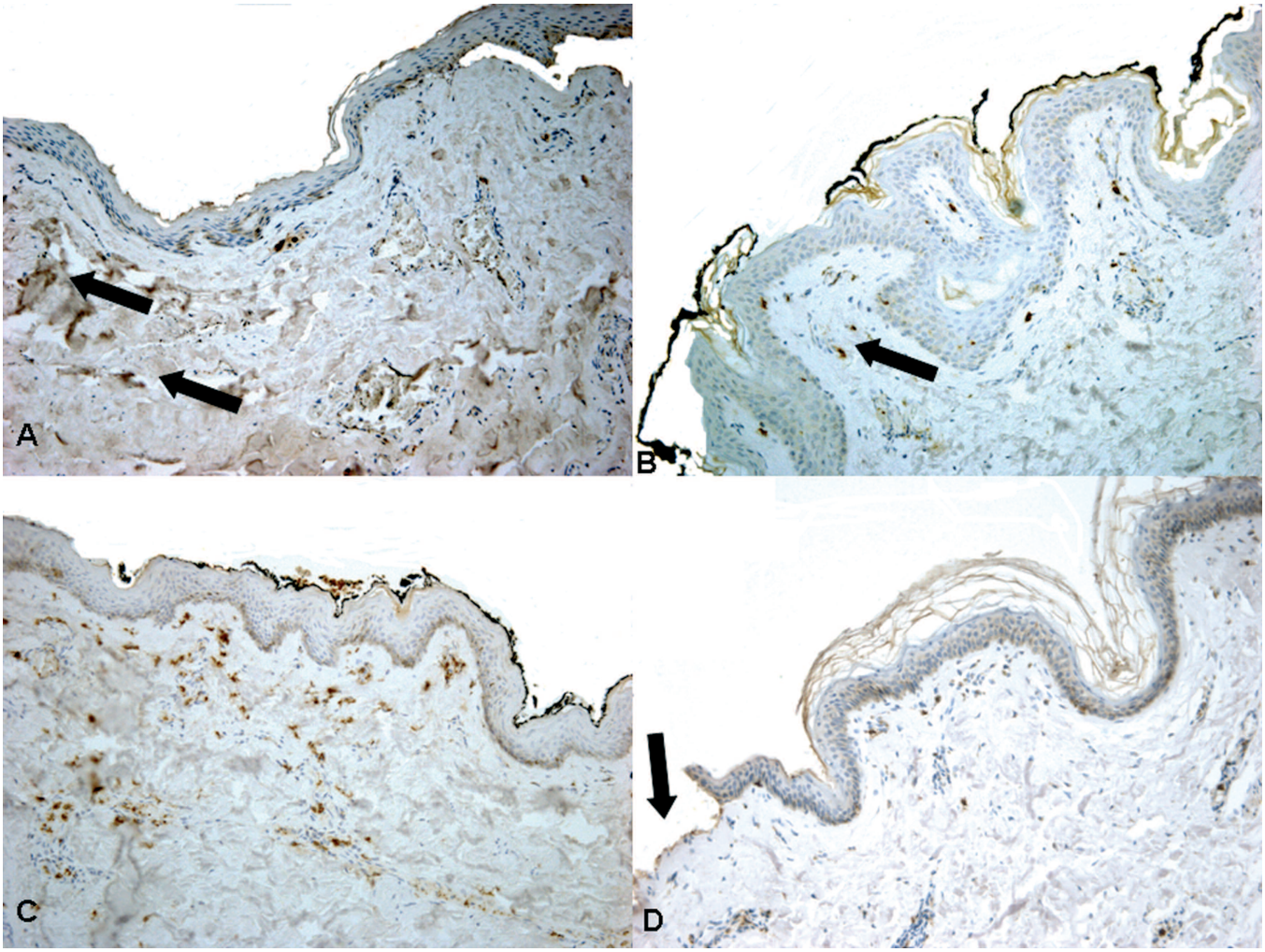

Fig. 2. A, B. CD15 reaction to demonstrate a few neutrophils near the vessels (early reaction). **C.** Randomly, sparse CD15 positivity of intravital hanging (late reaction). **D.** Removal of epidermal fragments (arrow) and light CD15 reaction near the vessel's sites. x 40

single mast cell degranulation has also been reported to appear early in vital response to trauma (Bonelli et al., 2003a,b). Based on our results, the presented evidence for mast cell activation, as judged by the extracellular release of tryptase in hanging mark's tissues, appears a very reliable parameter in distinguishing vital and post – mortem hanging, even when victims survived for a very short period and time is insufficient for other reactions to develop and become reliable to distinguish from lesions of post mortem origin.

In forensic practice, it is always necessary to distinguish between suicidal or accidental hanging and simulated or apparent hanging. Traditionally, rope burns caused by friction of rope against skin leading to blister formation, hemorrhagic bruises, and ecchymotic skin ridges are considered ante-mortem features. Similarly, hemorrhages in the cutaneous and subcutaneous tissues and in the deep soft parts, tissue ruptures with hemorrhages, hyperemia of the skin confined to the peripheral zones, incipient inflammatory reactions around the lesions are thought to indicate vital hanging (Janssen, 1984; Pollak and Mortinger, 1985; Mohanty et al., 2003). Many other vitality reactions have been described in connection with hanging: congestive hemorrhage in the conjunctiva of the eyes, petechiae of face skin, contusion of fatty tissue and emulsification of fat cells, swelling of the nerve axis cylinders and nerve endings, hemorrhages in the cervical limphonodes, hemorrhage within the point of attachment of neck muscles (especially the sternocleidomastoid muscle), segmental or discoid fragmentation of the muscle fibers with loss of the sarcoplasmatic cross-striation, and

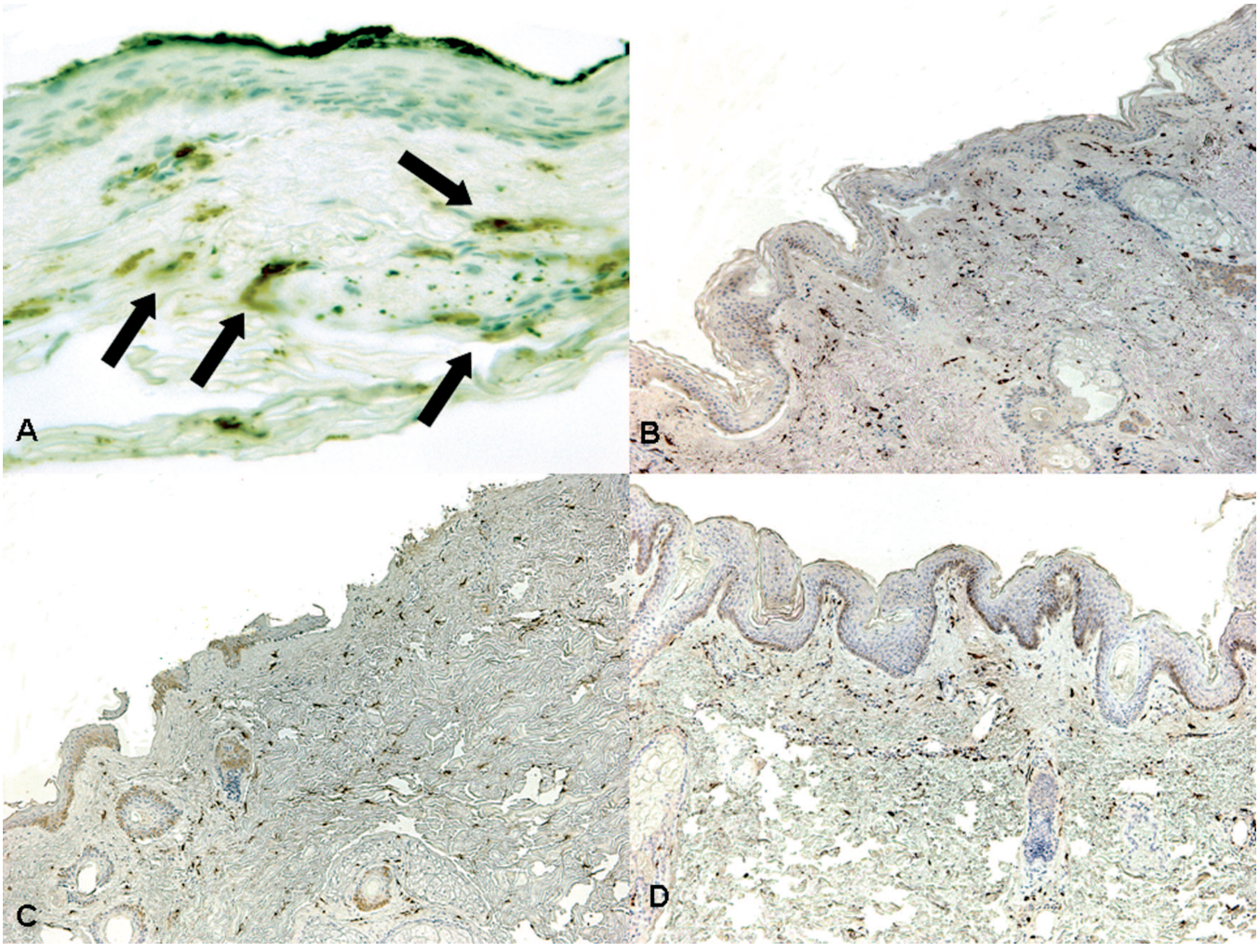

Fig. 3. Mast cells tagged by tryptase reaction. **A.** intense mast cells positivity and halo around the cells (arrows) especially near the epidermis. **B.** Mast cells appeared numerously, especially near the epidermis. **C.** Total detachment of the epidermis and sparse tryptase positivity of intravital hanging. **D.** Mast cells were scattered in the dermis, especially along the blood vessels and in the periglandular stroma to denote the intense tryptase positivity. A, x 80; B, D, x 40; C, x 20

hemorrhage accompanying laryngeal fracture (Kleiber et al., 1982; Janssen, 1984; Maxeiner, 1987; Sigrist et al., 1997; Sharma et al., 2008). However, these vital reactions are often absent in hanging, and furthermore, they can also be produced postmortem (Aghayev et al., 2005). Also the ecchymotic skin ridges, which are frequently found between the ligature turns in cases of dual or multiple loop ligature, can be produced many hours after death, even outside the hypostatic area (Pollak and Mortinger, 1985). The application of selective immunohistochemical techniques in determining wound age and vitality (Betz, 1994; Grellner et al., 1998, 2005; Hernàndez-Cueto et al., 2000; Grellner, 2002; Kondo, 2007; Bai et al., 2008; Takamiya et al., 2008) opened up a new field of investigation in the issue of ligature marks by forensic pathologists (Fineschi et al., 1998; Grellner and Madea, 2007). However, the majority of scientific studies in this field deal with dermal injuries due to sharp force. Studies on dermal injuries due to blunt force or other types of trauma are almost completely missing (Fechner et al., 1993; Fineschi et al., 2005). These data might be applicable to cases of manual strangulation too, as a previous study demonstrated with different immunohistochemical markers (Fieguth et al., 2003). Besides routine histological techniques, the immunohistochemical investigation of many bioactive substances essentially involved in skin wound healing, may give a substantial contribution to manual or ligature marks vitality estimation. In cases with even minimal trauma and no significant muscle damage the lack of any immunohistochemically-detectable changes allows no

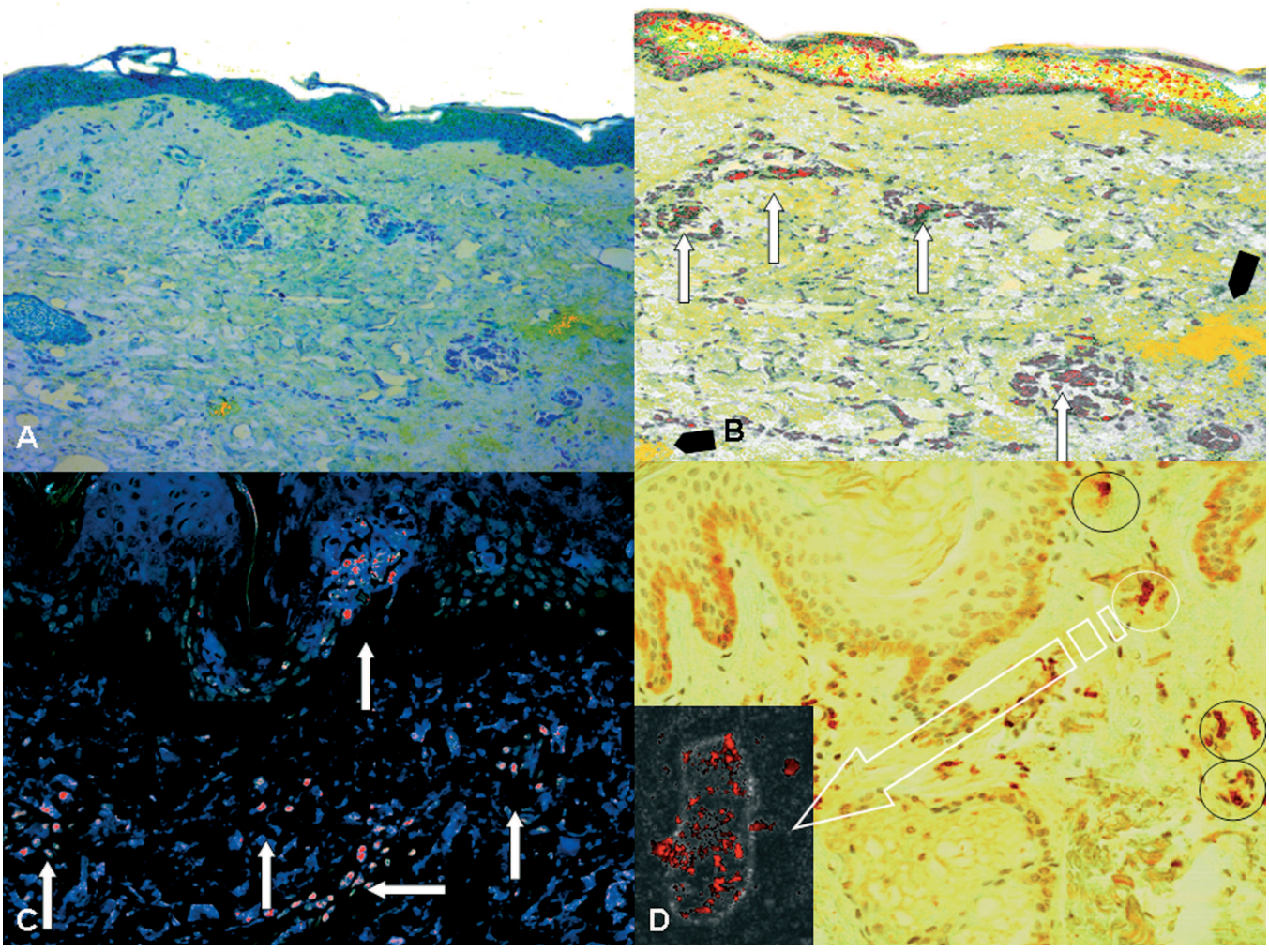

Fig. 4. Confocal laser scanning microscope. **A.** In the dermis, randomly, scattered IL-15 positivity (yellow reactions) of intravital hanging specimen. **B.** Double labelling of intravital hanging specimen: antibody CD15 labelled neutrophils (red) and anti IL-15 antibody (yellow). **C.** CD15 positivity is demonstrated by neutrophils red labelled. **D.** Mastcells reaction near the epidermis with tryptase halo around the cells: degranulating mastcells as intravital indicator are shown in the insert (tryptase in red and cellular membrane in white). A, B, x 40; C, D, x 100; insert, x 300

differentiation between vital or post-mortem infliction (Grellner et al., 2007).

We conclude that both tryptase and IL-15 can moving up to complement the CD 15-based determination of ligature marks vitality with the accuracy needed for forensic purposes. This fact especially applies to soft marks which are particularly difficult to evaluate on the basis of gross examination and of conventional histological studies.

References

Badolato A., Ponzi A.N., Millesimo M., Notarangelo L.D. and Musso T. (1997). Interleukin 15 (IL-15) induces IL-8 and monocyte chemotactic protein 1 production in human monocytes. Blood 7, 2804-2809.

Bai R., Wan L. and Shi M. (2008). The time-dependent expressions of IL-1beta, COX-2, MCP-1 mRNA in skin wounds of rabbits. Forensic Sci. Int. 175, 193-197.

Betz P. (1994). Histological and enzyme histochemical parameters for the age estimation of human skin wounds. Int. J. Leg. Med. 107, 60-68.

Betz P. (1995). Immunohistochemical Parameters for the Age Estimation of Human Skin Wounds. A review. Am. J. Forensic Med. Pathol. 16, 203-209.

Bonelli A., Bacci S., Vannelli G.B. and Norelli G.A. (2003a). Immunohistochemical localization of mast cells as a tool for the discrimination of vital and postmortem lesions. Int. J. Legal Med. 117, 4-18.

Bonelli A., Bacci S. and Norelli G.A. (2003b). Affinity cytochemistry analysis of mast cells in skin lesions: a possible tool to assess the timing of lesions after death. Int. J. Legal Med. 117, 331-334.

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

Fieguth A., Franz D., Lessig R. and Kleemann D.J. (2003). Fatal trauma to the neck: immunohistochemical study of local injuries. Forensic Sci. Int. 175, 193-197.

Fineschi V., Dell'Erba A.S., Di Paolo M. and Procaccianti P. (1998). Typical homicide ritual of the Italian Mafia (incaprettamento). Am. J. Forensic Med. Pathol. 19, 87-92.

Fineschi V., D'Errico S., Neri M., Panarese F., Ricci P.A. and Turillazzi E. (2005). Heat stroke in an incubator: an immunohistochemical study in a fatal case. Int. J. Legal Med. 119, 94-97.

García-Ramallo E., Marques T., Prats N., Beleta J., Kunkel S.L. and Godessart N. (2002). Resident Cell Chemokine Expression Serves as the Major Mechanism for Leukocyte Recruitment During Local Inflammation. J. Immunol. 169, 6467-6473.

Grellner W. (2002). Time-dependent immunohistochemical detection of proinflammatory cytokines (IL-1beta, IL-6, TNF-alpha) in human skin wounds. Forensic Sci. Int. 130, 90-96.

Grellner W. and Madea B. (2007). Demands on scientific studies: Vitality of wounds and wound age estimation. Forensic Sci. Int. 165, 150-154.

Grellner W., Dimmeler S. and Madea B. (1998). Immunohistochemical detection of fibronectin in postmortem incised wounds of porcine skin. Forensic Sci. Int. 97, 109-116.

Grellner W., Vieler S. and Madea B. (2005). Transforming growth factors (TGF-alpha and TGF-beta1) in the determination of vitality and wound age: immunohistochemical study on human skin wounds. Forensic Sci. Int. 153, 174-180.

Hernàndez-Cueto C., Girela E. and Sweet D.J. (2000). Advances in the diagnosis of wound vitality: a review. Am. J. Forensic Med. Pathol. 21, 21-31.

Janssen W. (1984). Forensic histopathology. Springer - Verlag Berlin Heidelberg pp. 214-217.

Kim M.H., Liu W., Borjesson D.L., Curry F.R., Miller L.S., Cheung A.L., Liu F.T., Isseroff R.R. and Simon S.I. (2008). Dynamics of neutrophil infiltration during cutaneous wound healing and infection using fluorescence imaging. J. Invest. Dermatol. 128, 1812-1820.

Kleiber M., Koops E., Puschel K., Gottberg J. and Brinkmann B. (1982). Pathology of hanging with special reference to vital reactions. Beitr. Gerichtl. Med. 40, 117-121.

Kondo T. (2007). Timing of skin wounds. Legal Med. 9, 109-114.

Leth P. and Vesterby A. (1997). Homicidal hanging masquerading as suicide. Forensic Sci. Int. 85, 65-71.

Maxeiner H. (1987). Local vital reaction following injuries of the neck, Z. Rechtsmed. 99, 35-54.

Mohanty M.H., Rastogi P., Kumar G.P., Kumar V. and Manipady S. (2003). Periligature injuries in hanging. J. Clin. Forensic Med. 10, 255-258.

Pelletier M. and Girard D. (2005). Interleukin-15 increases neutrophil adhesion onto human respiratory epithelial A549 cells and attracts neutrophils *in vivo*. Clin. Exp. Immunol. 14, 315-325.

Pollak S. and Mortinger H. (1985). Findings in intermediate ridges of strangulation marks and their value as signs of vitality. Arch. Kriminol. 175, 85-94.

Scapini P., Lapinet-Vera J.A., Gasperini S., Calzetti F., Bazzoni F. and Cassatella M.A. (2000). The neutrophil as a cellular source of chemokines. Immunol. Rev. 177, 195-203.

Sharma B.R., Harish D., Sharma A., Sharma S. and Singh H. (2008). Injuries to neck structures in deaths due to constriction of neck, with a special reference to hanging. J. Forensic Legal Med. 15, 298-305.

Sigrist T., Germann U. and Markwalder C. (1997). Using muscle histology for assessment of vitality in hanging. Arch. Kriminol. 200, 107-112.

Takamiya M., Fujita S., Saigusa K. and Aoki Y. (2008). Simultaneous detection of eight cytokines in human dermal wounds with a multiplex bead-based immunoassay for wound age estimation. Int. J. Legal Med. 122, 143-148.

Accepted May 26, 2010