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ORIGINAL ARTICLE

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Immunohistochemical localization of mast cells as a tool for the discrimination of vital and postmortem lesions

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Abstract In wounds that are inflicted at least 60 min before death, histamine levels can increase up to 100%. This functional effect might have a morphometric counterpart. Mast cells play a crucial role in acute inflammatory reactions and in the healing process of wounds. Therefore, the density of these cells was immunohistochemically assessed in tissue from 20 healthy controls (Group 1), 20 vital skin lesions (Group 2) (age range: a few seconds to 1 h), and 20 postmortem lesions (Group 3). A piece of skin close to the vital lesion was also obtained from the homolateral part of the body (Group 4). Mast cell density was significantly higher at the level of the vital lesions (11.28±2.44) than elsewhere (healthy controls 7.66±1.27, postmortem lesions 4.13±1.46, skin close to the vital lesions 4.88±1.59). No differences were found between the values assessed in the skin samples close to the vital lesions and in those in the postmortem lesions. Therefore, mast cell richness in the vital lesions exhibited a proportional morphological correlation with previously detected histamine values in cutaneous vital lesions. These results suggest that the detection of mast cells with immunohistochemical techniques can lead to a high level of discrimination (based on statistical data) between antemortem and postmortem lesions. This method could also be used to ascertain the vitality of lesions.

Keywords Immunohistochemistry · Mast cells · Postmortem lesions · Vital lesions

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Introduction

In many cases, the forensic pathologist is not able to arrive at a diagnosis of the vital origin of some wounds. In forensic case work investigations, there are many situations where the vital or non-vital origin of the wound is able to be established using only macroscopical examination. Other circumstances require additional studies to obtain a more exact diagnosis of tissue vitality. Sensitive methodology must be employed so as to detect the numerous substances involved in the first steps of an inflammatory reaction [1]. During the past 50 years, the development of histochemistry, enzymology, and biochemistry, and the application of these studies to the diagnosis of wound vitality have allowed for a partial solution to the problem [1, 2, 3, 4, 5].

It is well known that histamine, an important vasoactive amine, participates in an acute inflammatory reaction [6]. Endogenous histamine is responsible for initiating vascular changes that involve vasodilatation and increased vascular permeability and other mechanisms are then required to maintain them [7].

In 1965 Fazekas and Virágos-Kis [8] observed that there was an increase in the free histamine content in marks caused by hanging. Their work encouraged a number of forensic pathologists to begin biochemical studies on the possible use of the histamine content in the skin to differentiate antemortem from postmortem wounds and to estimate lesion vitality [9, 10, 11]. In particular, Berg and Bonte demonstrated that the histamine levels in vital skin wounds inflicted at least 60 min before death could increase up to 100% [12].

We hypothesized that these biochemical events might also have a histologic correlation: a quantitative alteration in the number of histamine secreting cells, such as mast cells (MC) [13, 14], could also take place.

We therefore carried out an in situ immunohistochemical study to compare the density of MC in vital wounds that had been inflicted from a few seconds to 1 h before death (according to hospital staff), and in postmortem lesions.

Material and methods

Protocol

A total of 80 lesions were examined and 20 tissue samples were taken during surgical treatment of wounds from patients who gave written consent after having been informed of the purpose of the sampling. The procedures were carried out with strict adherence to Italian law and in accordance with the ethical guidelines of the Italian National Medical Council.

A protocol was designed and the biopsy samples were divided into four different groups as follows:

- Group 1 (n=20) Consensually donated biopsies of clinically healthy skin that had been excised at surgery. These samples were used as positive controls.
- Group 2 (n=20) Vital skin lesions (surgical wounds, lacerations and abrasions) from injuries that had been sustained before death. The age of the examined samples ranged from a few seconds to 1 h. Vital wounds were classified into four different subgroups according to the time elapsed between the moment of injury and the estimated moment of death (survival time) as follows: <5 min (n=9), <15 min (n=6), <30 min (n=3) and <60 min (n=2).
  The age of the tissue donor ranged from 22 to 79 years, mean age: 48.3 years and there were 19 men and 1 woman.
- Group 3 (n=20) Post-mortem lesions that had been obtained during routine autopsies.
- Group 4 (n=20) Control pieces of skin, close to the vital lesions, that had been obtained from the homolateral part of the body.

All the autopsies were performed at the Department of Anatomy, Histology, and Forensic Medicine, a section of Forensic Medicine at the University of Florence (Italy). The cadavers were routinely kept at +4°C until autopsy. The time between death and autopsy was 24 h.

Histochemistry and morphometry

After fixing in Bouin or Carnoy's fluid [15], all of the specimens were dehydrated in ethanol, embedded in paraplast and five sec-

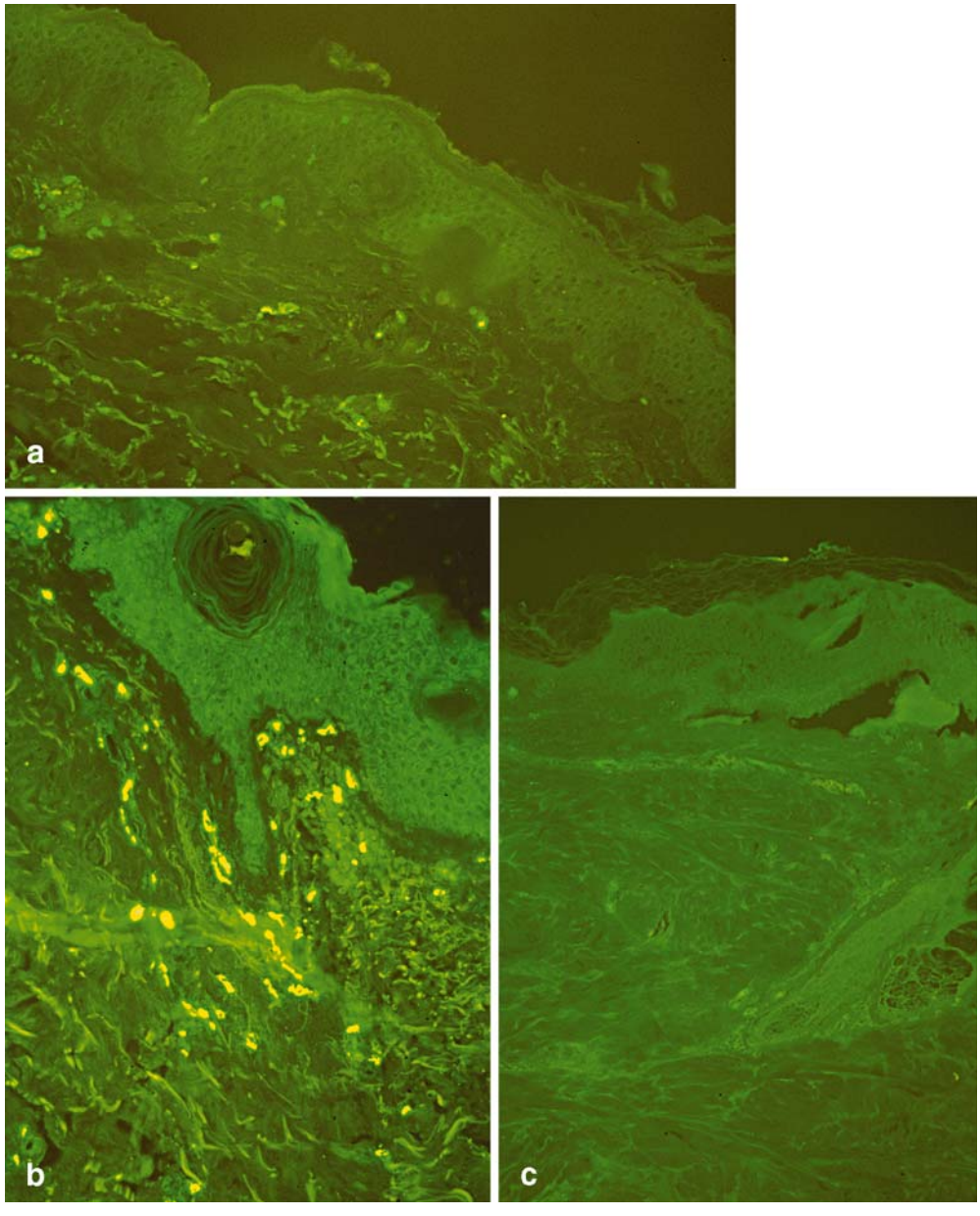

Fig. 1a–c Mast cells after antitryptase immunohistochemistry. Bouin's fixation, magnification ×100. **a** Positive control, **b** a vital lesion, **c** a post-mortem lesion

tions were cut per block. Before bright field microscopy, sections were stained with hematoxylin eosin and 0.1% toluidine blue at pH 4.3 (Carlo Erba, Milan, Italy) [16]. Before fluorescence microscopy was carried out, sections were stained with an indirect immunohistochemical method for tryptase and chymase antibodies (Chemicon, Rome, Italy) [17, 18]. They were also stained with fluorescein isothiocyanate-labeled avidin (Sigma, Milan, Italy) [19] diluted to 1:400 in 1% albumin (Sigma, Milan, Italy). Primary mouse monoclonal antibodies, anti-tryptase and anti-chymase were diluted to 1/1,000 and applied at 4°C. All primary antibodies were diluted with phosphate-buffered saline (PBS). Anti-mouse polyclonal goat fluorescein isothiocyanate-labeled antibodies were used as secondary antibodies (Sigma, Milan, Italy) [20]. Fluorochrome stained sections were examined with a Nikon Microphot-Fx (Tokyo, Japan) microscope equipped for epi-fluorescence. Immuno-staining specificity was tested by omitting the first antibody or substituting it with an irrelevant one.

During the morphometric studies, sections were taken from each tissue block that had been stained with fluorescein avidin. The mast cells were then counted by one of the authors (SB) and counted again by another (BV), after staining with anti-tryptase and anti-chymase antibodies (magnification×200). In order to keep approximation and assumptions to a minimum, the density of immuno-labeled cells was expressed as the number of stained cells per unit section surface area. All the cells were counted, the surface area was measured in one section per biopsy and the number of cells per square millimeter of section surface area was computed.

Statistical analysis

The statistical significance of the differences in the means of each experimental group was determined by analysis of variance. Mean differences were considered significant when $P<0.05$. Results are expressed as the mean and standard deviation. Analyses were performed using a StatView 512+ (Abacus, Concepts, Berkeley, Calif.) [21].

Results

Distribution and number of mast cells

The 80 specimens included in the study did not have any histologic abnormality [1].

Using light microscopy, all the mast cells (MC) in the specimens (however fixed) had metachromatically stained purple with toluidine blue, pH 4.3. At fluorescence microscopy, these cells had also been labeled by fluorescent avidin and immunostained for tryptase and chymase (Fig. 1a–c). The nuclei of all the cells were round or oval. MC were scattered in the dermis, especially along the blood vessels and in the periglandular stroma (Fig. 1a–c).

In the dermis of the vital and postmortem lesions and in the skin close to the vital lesions, the mean mast cell densities were 11.28±2.44, 4.13±1.46 and 4.88±1.59, respectively. Mast cell density in the vital lesions was significantly higher ($P<0.001$) when compared with the other examined groups. When the mast cell density results of the postmortem lesions and of the skin close to the vital lesions were compared to the controls (7.66±1.27), significant differences were found ($P<0.001$). In contrast, no significant differences were found in either the detected values of the skin close to the vital lesion or in the postmortem lesions (Fig. 2).

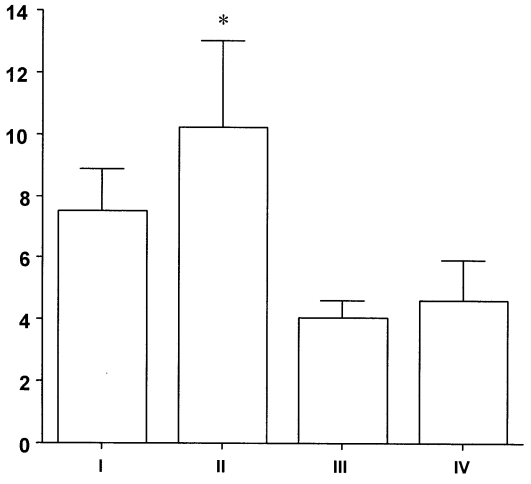

Fig. 2 Mast cell density (mean±SD) in all the studied groups (significance group II vs groups I, III, and IV $P<0.001$, group I vs groups III and IV $P<0.001$. Only those comparisons that yielded significant differences are mentioned)

Effects of fixation on staining and immunohistochemical properties of MC

Tryptase immunoreactivity was detected in all of the cells stained by fluorescent avidin fixed either with Bouin or Carnoy's fluid. Some MC, in the dermis of all the examined groups were immunostained for chymase upon fixation with Carnoy's fluid. Since the percentages of all these immunostained cells varied a greatly from case to case, these counts were omitted.

Discussion

A wound is defined as a break in the continuity of the soft parts of the body or damage to any part of the body caused by violence or trauma through the application of mechanical force [22]. These circumstances determine a cascade of chemical and morphological events, which when taken together, contribute to the so-called acute inflammatory reaction within the tissue [1]. The acute reaction is a complex mechanism which is not completely understood but consists of several apparently different processes. The last step is the destruction of noxious stimuli and the start of tissue repair [1].

Histamine is released into the cellular infiltrate by mast cells (MC) and basophils. Since this substance plays a crucial role in the early phases of any inflammatory reaction [1], many studies have been carried out to develop methods that can distinguish the content of this amine in vital and postmortem lesions [9, 10, 11]. In some other studies, it has been suggested that an increase in the mast cell degranulation index might be considered a sign of vitality [23], when correlated to histamine values in antemortem wounds. Berg and Ebel [24] described massive degranulation with a survival time of 2–4 h even if degranulation of single mast cells has also been reported to appear much earlier.

As a result of this study, detailed information was obtained on the number and distribution of MC in vital cutaneous and postmortem lesions. Based on our samples, we have concluded that the density of MC at the vital lesion is significantly higher ($P<0.001$) than in healthy controls, and in postmortem lesions. Furthermore, no significant differences were found between the density of MC in the skin close to the vital lesion and in postmortem lesions.

Although the present study is mainly morphometric in scope and does not directly explore the functional aspects, our results may provide some insight into histamine secretion modifications in vital and postmortem lesions [12] as morphology is generally affected by long-lasting functional changes.

The presence of unsegmented nuclei and intense tryptase immunoreactivity in all of the cells labeled using avidin, most probably indicates that all of the basophilic cells observed were MC and not basophilic granulocytes [25]. Tryptase and avidin immunolabeling can circumvent problems in labeling MC with basic dyes after fixation in formaldehyde-based fluids such as Bouin's fluid [26, 27]. Only Carnoy's fluid allowed for good conservation of chymase immunoreactivity and the usefulness of Carnoy's fluid for this purpose has already been recognized [17, 28].

The finding that the MC of the skin were heterogeneous for the content of neutral protease, e.g., tryptase and chymase, was not surprising [17, 28]. This may have indicated that subtypes of MC were present in this region. All the MC were tryptase positive and only some were chymase negative, as reported elsewhere [17, 28]. This heterogeneity was also present in our vital skin samples.

These findings are interesting from a physiologic point of view.

Slight degranulation occurs at any time in the MC in the skin [29, 30]. Therefore, it is reasonable to hypothesize that low concentrations of histamine and other mediators released by these cells, even under normal conditions, may be involved in the homeostasis of the skin and in the recruitment, differentiation and function of many cells, including MC [29, 30].

When a local stimulus such as an injury occurs, histamine release by MC increases [12, 13, 14]. It is maintained by a high efflux of precursors in the damaged zone [14], with respect to normal conditions. The recruitment of MC is generally paralleled by the expression of monocyte chemoattractant protein 1 (MCP-1), but not by other chemokines [14].

Death causes the cessation of blood flow and thus of any oxygen transport. The lack of oxygen and of blood-borne oxidizable substances deprives the cells of the major source of energy for the synthesis of adenosine triphosphate. This uncouples oxidative phosphorylation that in turn is further hampered by the imminent malfunction of the cellular membranes. As a result, there is an accumulation of calcium. The Ca^{2+} ion is a powerful inhibitor of oxidative phosphorylation but many enzymes retain their ability to function. However, even if they are still active, the activity is no longer coordinated [31]. This last fact may have been confirmed by the detected mast cell heterogeneity found in the vital skin in all of the experimental groups. Therefore, the altered activity of the mast cells mediators, especially low levels of tryptase, and consequently, the moderate capacity of the MC to secrete histamine [32] may be followed by a decrease in precursor efflux [14]. This is in accordance with the above mentioned data and other facts (different concentrations or chemical structures of local factors, such as complement component C3A or C3A des Arg [33] or low secretion of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα that are definitely involved in the inflammatory processes, [34]).

These biological mechanisms should explain the increase in cellular density in our positive controls with respect to the two other groups examined and the richness of MC at the vital lesions as compared to the controls.

A previous study analyzed the number of MC in vital and postmortem lesions in rats and in humans. Zhong and Zhen found no significant differences between the number of mast cells in intact skin and those in wounds in their sampling [31]. We are unable to explain this but perhaps differences in technical and morphometric methods can partly explain the discrepancies.

In conclusion, the detection of MC using immunohistochemical techniques can lead to a high level of discrimination between ante and postmortem wounds. This method (based on statistical data) might also be used to establish the vitality of lesions. Experiments to test this possibility are currently underway.

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