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ORIGINAL ARTICLE

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Early increase in TNF-alpha-containing mast cells in skin lesions

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Abstract We have evaluated the numbers and immunohistochemical positivity for tumor necrosis factor (TNF) alpha of the mast cells in (a) 40 skin samples collected at autopsy from subjects who had survived for a few seconds to 1 h, (b) 10 samples of post-mortem skin lesions and (c) 10 surgical biopsies of healthy skin. Sections were treated with fluoresceinated avidin, to tag mast cell granules, followed by indirect immunohistochemistry for TNF-alpha with polyclonal primary and rhodaminated secondary antibodies. We could confirm a progressive increase in mast cell numbers, which became significant 1 h after trauma. More important, the fraction of mast cells positive for TNF-alpha increased progressively in the same time period and became significantly higher than controls in specimens collected more than 15 min after trauma. Samples from post-mortem lesions had significantly fewer mast cells and fewer TNF-alpha-positive cells than any other group of samples. The results suggest that mast cells are quickly recruited to an injured site in response to trauma and up-regulate their TNF-alpha content, which can play an early role in directing tissue response to injury. The forensic pathologist can take advantage from this behavior of mast cells for the evaluation of the timing of early vital lesions.

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Introduction

The evolution of human skin lesions with time, and therefore, the relationships between the aspect of lesions and the time from trauma to death, is a major problem in forensic medicine, which is addressed on the basis of the normal wound healing process [1, 2]. Histamine levels in skin vary appreciably early after wounding [3, 4]; in particular, they increase significantly between 5 min and 3 h after trauma and decrease afterwards until 24 h [5]. Mast cells are a major source of tissue histamine, and we have previously demonstrated on autoptical skin specimens that the number of mast cells within 2 cm of lesions increases progressively with time up to a maximum within 1–3 h and decreases thereafter, becoming less than in the controls 6 h after trauma [6].

Tumor necrosis factor (TNF) alpha is a multifunctional, pro-inflammatory cytokine involved in the regulation of tissue homeostasis and local immune responses. The biochemical determination of the TNF-alpha content in tissue has been proposed as a possibly useful tool for the estimation of the vitality at the moment of lesion and of wound age, in particular, in the early post-traumatic interval before the onset of leucocyte reactions [7, 8]. Mast cells are a source of TNF-alpha [9, 10], together with other cell types [11–14], and TNF-alpha in turn can stimulate the secretion of histamine by mast cells [15]. Because of these interrelationships between TNF-alpha, mast cells and trauma, we have evaluated mast cell numbers and immunohistochemically detectable TNF-alpha content in skin wounds within 1 h after trauma with two aims: improving the knowledge on the behavior and possible role of mast cells after wounding and, if possible, expanding the available methods to estimate the time interval between wounding and death in autopsy specimens.

Material and methods

Tissue specimens

Control samples (n=10) were taken from the disease-free border of surgical specimens. They consisted in skin strips less than 1-cm wide, removed for plastic reasons from cutaneous areas appearing normal at inspection. Fifty more samples were obtained at autopsies performed at the Department of Anatomy, Histology and Forensic Medicine, section of Forensic Medicine, of the University of Florence (Italy). Corpses were routinely kept at +4°C from the moment they arrived at the morgue until autopsy. The time between death and autopsy was 24–48 h. The samples were divided into groups of ten as follows:

- Group 1: biopsies of clinically healthy skin, excised at surgery and used as controls.
- Groups 2–5: samples of vital skin lesions (surgical wounds, lacerations and abrasions), with different survival times between injury and death as estimated from the witness, police and emergency and hospital medical care records, as follows:
  - Group 2: time between lesion and death no more than 5 min. Subjects were seven men and three women, aged between 25 and 66 years (mean age 46.5 years).
  - Group 3: time between lesion and death 6–15 min. Subjects were six men and four women, aged between 37 and 79 (mean age 60.5 years).
  - Group 4: time between lesion and death 16–30 min. Subjects were eight men and two women, aged between 31 and 60 (mean age 43.3 years).
  - Group 5: time between lesion and death 31–60 min. Subjects were nine men and one woman, aged between 19 and 77 (mean age 57.1 years).
- Group 6: samples of post-mortem lesions, used as further controls. Subjects were six men and four women, aged between 15 and 86 (mean age 57. 1 years).

Histochemistry and morphometry

The specimens were prepared following a previously published method [6]. Briefly, they were fixed in Carnoy's fluid and embedded in paraplast. Sections were stained with haematoxylin and eosin, with an indirect immunofluorescence method for TNF-alpha and with fluoresceinated avidin (1:400, 1 h at 37°C; Sigma, Milan, Italy) to selectively tag the mast cells [16]. For TNF-alpha immunostaining, primary mouse monoclonal antibody (1:50, overnight at 4°C; Sigma) was followed by rhodaminated secondary goat polyclonal antibody (1:32, 1 h at 37°C; Sigma). Omission of the primary antibody was used to check the specificity of the immune reaction.

Statistical analysis

Mast cells were counted in at least five microscopical fields per biopsy at magnification ×200 (field area 0.8 mm^2); TNF-alpha-positive mast cells were counted separately from those positive only for avidin. One section was used for each biopsy. A similar surface area was observed in each case, no fragment extended beyond 20 mm from the lesion and the numbers of samples were relatively high; therefore, we assumed the examined samples as representative of the tissue as a whole and therefore proceeded to comparisons among the different experimental groups. The number of cells per unit section surface area was subjected to analysis of variance (ANOVA). The numbers of TNF-alpha-positive vs TNF-alpha-negative mast cells were also analysed by non-parametrical chi-square test. All tests were applied two-tailed. For multiple comparisons, i.e. among the several groups of patients, the significance limit was set at p=0.001; for comparisons between pairs of values, i.e. TNF-alpha-positive vs TNF-alpha-negative cells and one experimental group against another, the significance limit was set at p=0.05. Mean values and standard deviation are given as results.

Results

The analysed specimens had no cell infiltrate in the tissue (Fig. 1). At fluorescence microscopy, mast cells as labelled by fluorescent avidin were round or oval; they were scattered in the dermis. (Fig. 2a). A progressive increase in mast cell numbers, which became significant in specimens 31 to 60 min after trauma, was found in vital lesions (Figs. 2c and 3), and some mast cells were stained for TNF-alpha in controls (Fig. 2b). The number of mast cells stained for TNF-alpha increased progressively and significantly with time and became significantly different

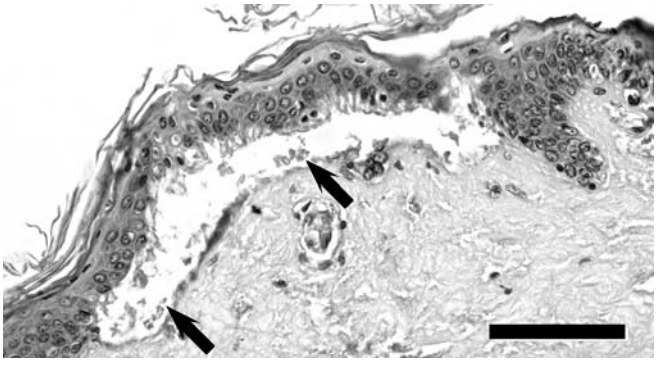

Fig. 1 Vital skin lesion, 31 to 60 min between injury and death. Traumatic detachment of epidermis from dermis led to the formation of a phlycten containing extravasated red blood cells (*arrows*). There is no inflammatory cell infiltrate. Haematoxylin and eosin, *bar*=100 μm

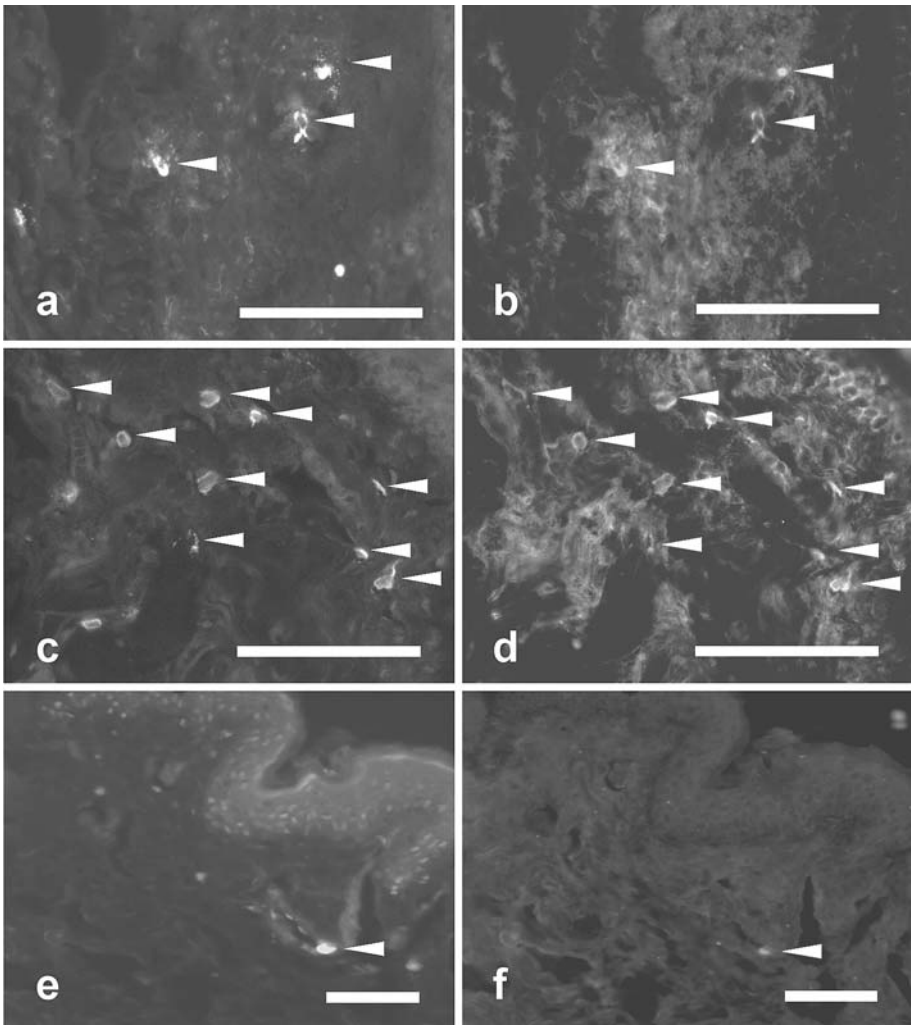

Fig. 2 Mast cells stained with fluorescent avidin in a control biopsy (**a**), in a lesion that occurred 31 to 60 min before death (**c**) and in a post-mortem lesion (**e**). Some of these cells (*arrows*) were also labelled for TNF-alpha: control biopsy (**b**), vital lesion that occurred 31 to 60 min before death (**d**) and post-mortem lesion (**f**). Fluorescence microscopy, *bar*=100 μm

from the controls when the survival time was more than 15 min after lesions (Figs. 2d and 4). The number of TNF-alpha-negative mast cells followed an opposite course (Figs. 2d and 4). Samples from post-mortem lesions had significantly fewer mast cells (Figs. 2e and 3) and fewer TNF-alpha-positive cells (Figs. 2f and 4) than any other group of samples. No immunostaining was seen in control sections not treated with primary antibodies.

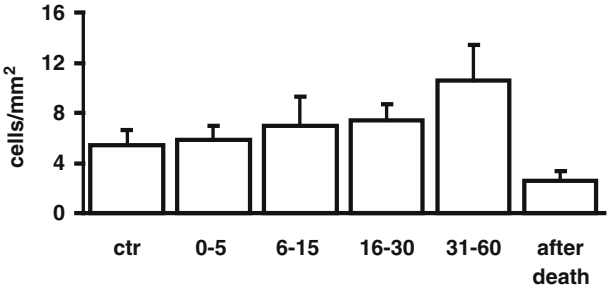

Fig. 3 Numbers of mast cells per mm^2 of skin section surface area. The differences among all groups of biopsies were significant ($p<0.001$, ANOVA). Values after 31–60 min from injury were significantly higher ($p<0.05$), and those after death significantly lower ($p<0.05$), than those of any other group. Means and standard deviations among microscopic fields are shown

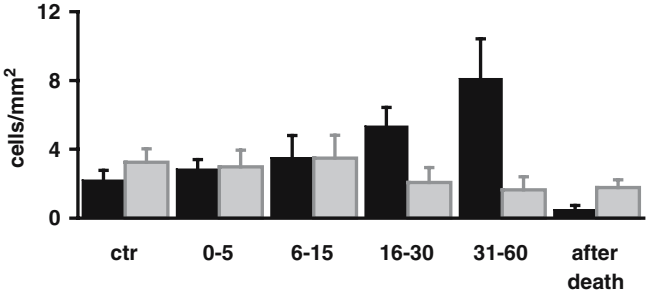

Fig. 4 Numbers of mast cells labelled for TNF-alpha (*black columns*) and unlabelled for this cytokine (*white columns*) per mm^2 of section surface area of skin. The differences among groups of biopsies were significant ($p<0.001$). In paired comparisons, a significant difference from controls was reached already in samples 16–30 min from lesions as well as in post-mortem lesions ($p<0.05$)

Conclusions

Since we had shown in a previous paper that the number of histochemically detectable mast cells varies with time within a few hours from skin wounding [6], we have now asked whether the analysis of mast cell subgroups might help to estimate survival within the first hour after wounding. This turned out to be the case, since increase in TNF-alpha-positive mast cells and a concomitant fall in TNF-alpha-negative mast cells were significant for 15 min after injury, earlier than when the total number of mast cells become significantly different from controls. The present findings stress the importance of TNF-alpha secreted by mast cells as an early mediator molecule in the response of wounding, since the observed modifications occurred long before any sign of inflammatory cell infiltration was detectable by routine tissue examination. Although it is often read that neutrophils are recruited to skin wounds within minutes [17, 18], articles providing original information [6, 19–21] and detailed reviews [22, 23] indicate appreciable neutrophil infiltration onsets only since at least 1 h or more after wounding.

In the mouse, immunohistochemical demonstration of TNF-alpha could be obtained in formalin-fixed, paraffin-embedded sections; the cytokine was shown to increase between 1 and 3 h after wounding [24]. TNF-alpha-positive cells were interpreted as keratinocytes and dermal neutrophils, which infiltrated the skin at 3 and 6 h after wounding. The authors [24] gave no indication as to whether neutrophils were the only cell positive for TNF-alpha apart from keratinocytes nor did they perform specific staining for mast cells. Therefore, it cannot be excluded that mast cells play a role in the early response to wounding even in the mouse.

Biochemical analysis had shown that TNF-alpha is up-regulated in the skin since 15 min upon injury, with a maximum after 60–90 min [7]. The present results show that mast cells can be major contributors to these modifications. In addition, the present results complete the work of Grellner [8], who analysed human skin specimens frozen and stored in liquid nitrogen, cryosectioned, air-dried and cold-acetone-fixed. In these conditions, TNF-alpha immunoreactivity was found upon injury within keratinocytes, in the dermal interstice and also in some dermal cells (as shown in Fig. 6 in the study of Grellner [8]). The present results show that at least part of dermal TNF-alpha-positive cells are mast cells and suggest that a large amount of immunohistochemically detectable TNF-alpha is lost with routine fixation and embedding procedures and that this loss occurs from the epidermis and from dermal deposits other than mast cell granules. In addition, the present results indicate that, apart from cryosections fixed in cold acetone [8], mast cell TNF-alpha can be immunolabelled in routinely fixed and stored tissue samples, which add value to the method that does not require either destruction of the samples (as for biochemical assays) or keeping the specimen continuously frozen without interruption from sampling to sectioning and analysing (as for immunohistochemistry on cryosections).

The results of this study suggest that mast cells can quickly modulate their content of TNF-alpha. On the basis of our data, we cannot say to what extent the results depend on synthesis of TNF-alpha by pre-existing mast cells or on the differentiation of TNF-alpha-rich mast cells from unlabelled precursors present in the dermis or influxing from blood. The inverse relationship between the number of TNF-alpha-positive and TNF-alpha-negative cells is in favour of the first hypothesis; the progressive increase in the total number of mast cells is in favour of the second one, so it seems reasonable to suggest that both mechanisms cooperate to cause the observed modifications.

The timing of injury in respect to death is a major challenge in forensic medicine, which has prompted to explore the possible usefulness of biochemical and histochemical analyses to this aim. The increase in tissue albumin, upon compensation for albumin directly deriving from hemorrhages, has been detected by liquid chromatography since 15 min after wounding in rats; this analysis requires tissue destruction, and it is not known whether the results may be relevant to humans [25]. By histochemistry, the expression of vascular endothelial growth factor was found to be significantly increased above baseline only after more than 12 h upon wounding in humans [21], so it seems to be of little use for shorter survival times. The expression of fibronectin and tenascin in the dermis may be of help at early time points (15 min or more) in the rat [26], but only fibronectin appears to be of help for humans at similar time points, although with limitations in terms of sensitivity and pattern of staining [27, 28]. Tenascin, on the contrary, may give information only for survival times of at least a couple of days [29].

Given the results of this study, the immunohistochemical analysis of TNF-alpha-containing mast cells can be proposed as complementary to other investigations to discriminate between vital and post-mortem wounds and to estimate the time interval between injury and death.

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