Int J Legal Med (2002) 116:87–91

ORIGINAL ARTICLE

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Immunohistochemical detection of chemokines in human skin wounds and its application to wound age determination

Received: 30 May 2001 / Accepted: 14 August 2001

Abstract Immunohistochemical studies on the time-dependent expression of the chemokines such as interleukin (IL)-8, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α were performed on 50 human skin wounds with different wound ages (group I 0–12 h, group II 1–4 days, group III 7–14 days and group IV 17–21 days). In the wound specimens with wound ages between 4 and 12 h, neutrophils mainly showed positive reactions for IL-8, MCP-1 and MIP-1α. With increasing wound ages, macrophages and fibroblasts were positively stained with anti-IL-8, MIP-1α and MCP-1 antibodies. Morphometrically, there was a similar distribution in the positive ratios of the inflammatory cells among IL-8, MCP-1 and MIP-1α. The positive ratios of each chemokine were very low in group I and a considerable increase of the positive ratios in each chemokine was observed in group II (mean ± standard error IL-8: 59.8 ± 2.1%, MCP-1: 42.4 ± 3.1% and MIP-1α: 50.4 ± 3.7%). Although the positive ratios for each chemokine gradually decreased according to the wound age, the mean positive ratios in groups III and IV were significantly higher than those in group I. From the forensic aspect, these chemokines are considered useful markers for wound age determination. Thus, ratios of > 50% for IL-8, > 30% for MCP-1 or > 40% for MIP-1α indicate a wound age of at least 1 day. Moreover, the combined investigation of these three chemokines can make wound age determination more objective and accurate.

Keywords Wound healing · Wound age determination · Interleukin-8 (IL-8) · Monocyte chemoattractant protein-1 (MCP-1) · Macrophage inflammatory protein-1α (MIP-1α)

This study was presented at the 1st World Wound Healing Congress, Melbourne, September 2000 and the 79th Annual Meeting of German Society of Legal Medicine, Essen, September 2000.

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Introduction

Wound healing is a complex but spatially and temporally controlled biological response, and it is generally composed of inflammatory, proliferative and maturation phases [8, 34]. It is well known that various kinds of biological substances such as growth factors, cytokines and adhesion molecules are closely related with the wound healing process [31, 41]. In particular, interleukin 1 (IL-1), IL-6 and tumor necrosis factor (TNF)-α play an important role in the promotion of inflammatory reactions and fibroblast growth factor, transforming growth factor and vascular endothelial growth factor mainly contribute to the formation of granulation tissue and angiogenesis. In forensic pathology, the expression of these biological substances in skin wounds was applied to wound age determination [3, 4, 5, 6, 7, 13, 14, 15, 16, 21, 26, 27, 35, 36, 37, 38].

One of the proposed functions of the chemoattracting cytokines, so-called chemokines is to promote leukocyte infiltration. Recently, many chemokines have been found and cloned [9] and in particular chemokines such as IL-8, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α are considered to be closely involved in wound healing [11, 17, 18]. The authors immunohistochemically examined the time-dependent expression of IL-8, MCP-1 and MIP-1α in human skin wounds.

Materials and methods

Materials

A total of 50 human skin wounds with wound ages ranging from a few minutes to 21 days were collected at forensic autopsies (De-

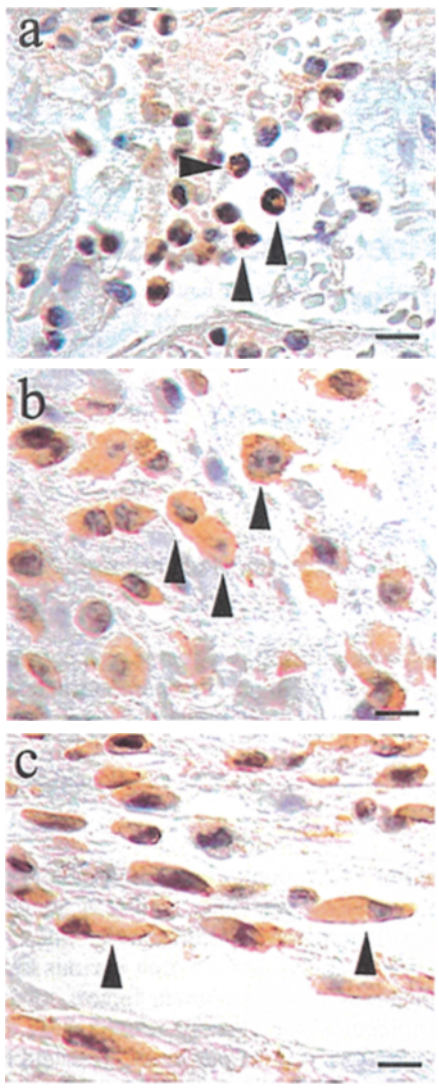

Fig 1 **a** 12-h-old skin wound with infiltration of neutrophils *(arrowheads)* showing positive reaction for IL-8 and MCP-1 and MIP-1α gave similar findings. **b** In this 4-day-old wound, phagocytic macrophages *(arrowheads)* are immunostained with anti-MCP-1 antibody and IL-8 and MIP-1α gave similar findings. **c** In this 14-day-old wound, spindle-shaped fibroblastic cells *(arrowheads)* were positively immunostained with anti-MIP-1α antibody and similar staining results were obtained for IL-8 and MCP-1 (bar = 10μm)

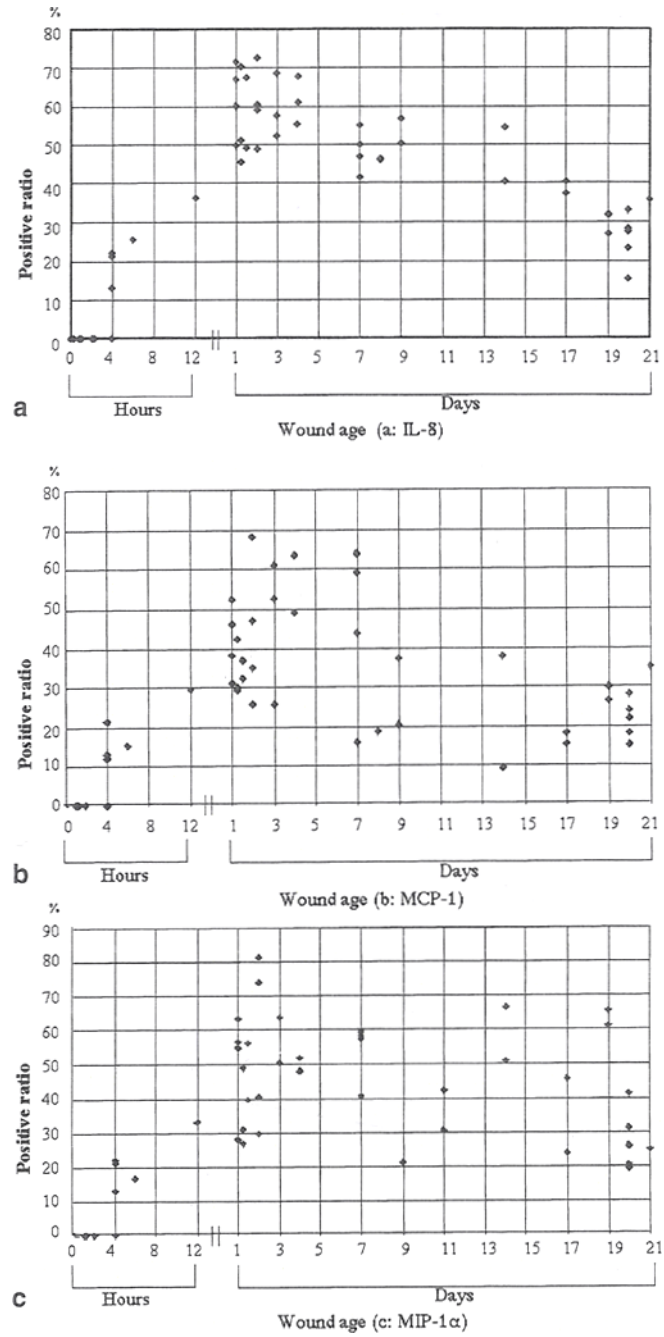

Fig. 2 Ratio of positive infiltrating cells in relation to the wound age, **a** IL-8, **b** MCP-1 and **c** MIP-1α

partment of Legal Medicine, University of Munich). Individual ages at death ranged from 7 to 77 years (mean age 48.8 years) and the post-mortem interval was less than 3 days in every case. None of the cases had suffered from apparent malnutrition, malignant diseases or metabolic disorders, and no substances such as cytostatic agents or glucocorticoids which may influence wound healing, had been administered for medical treatment. Wound specimens were classified into four groups according to the wound ages as follows; group I 0–12 h (n = 11), group II 1–4 days (n = 19), group III 7–14 days (n = 9) and group IV 17–21 days (n = 11). Non-wounded skin samples from the same individuals were also taken as controls.

Immunohistochemistry

Immunohistochemical analysis was performed according to previous studies [27, 28]. Briefly, after making paraffin-embedded sections, rabbit anti-human IL-8, – MCP-1 or – MIP-1α polyclonal antibodies (Genzyme, USA) were incubated at 4 °C overnight as primary antibody. Positive reactions were then visualised with diaminobenzidine using the Envision+™ detection kit (Dako, Japan).

Morphometrical analysis

Morphometrical analysis was performed according to previous studies [27, 28] in order to semi-quantitatively evaluate the immunohistochemical findings by two different investigators (TK, DG).

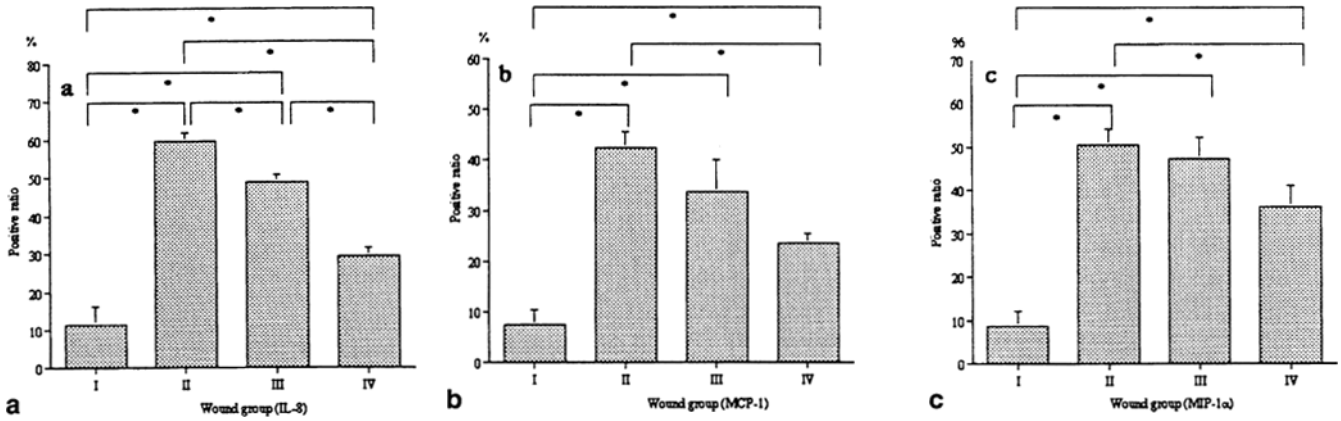

Fig. 3 Mean values and standard errors in each wound group for **a** IL-8, **b** MCP-1 and **c** MIP-1α. (* a significant difference was observed by the Mann-Whitney U-test: $p < 0.05$)

Statistical analysis

In each group, the mean values of the IL-8, MCP-1 or MIP-1α positive ratios and standard errors (SE) were calculated. Statistical significance was evaluated by the Mann-Whitney U-test.

Results

Immunohistochemistry

In the non-wounded specimens, all three chemokines were detected in the keratinocytes, sweat gland cells and endothelial cells. In the wound specimens with ages of 4–12 h, polymorphonuclear cells, probably neutrophils were mainly observed at the wound site, and some of them showed IL-8, MCP-1 or MIP-1α positive reactions in the cytoplasm (Fig. 1 a). With increasing wound age, round-shaped mononuclear cell (probably macrophages) infiltration was dominant over neutrophil infiltration and then the migration of spindle-shaped fibroblasts with granulation tissue formation and angiogenesis were also observed. IL-8, MCP-1 and MIP-1α were localised in the cytoplasm of the macrophages (Fig. 1 b) and fibroblasts (Fig. 1 c).

Morphometrical analysis

There was a similar distribution in the immuno-positive ratios of IL-8, MCP-1 and MIP-1α (Fig. 2). Although positive ratios for each individual chemokine were very low in group I, the ratios for each chemokine considerably increased in group II (mean ± SE: IL-8 59.8 ± 2.1%, MCP-1 42.4 ± 3.1% and MIP-1 50.4 ± 3.7%). The maximum ratios in IL-8, MCP-1 and MIP-1α were 72.6, 68.6 and 81.8% in a 2-day-old wound, respectively. Thereafter, the positive ratios gradually decreased in groups III and IV. For IL-8, there were significant differences among the four groups (Fig. 3 a) and in particular 15 out of 19 wounds in group II had IL-8 positive ratios > 50%. On the other hand for MCP-1 and MIP-1α significant differences were found between group I and the other three groups and between groups II and IV. However, there were no significant differences between groups II and III and between groups III and IV (Fig. 3 b, c).

Discussion

The production of IL-8, MCP-1 and MIP-1α is induced by bacterial or viral infection and other inflammatory cytokines like IL-1 and TNFα [32]. The biological role of IL-8 is chemotaxis for polymorphonuclear cells [32], T lymphocytes [30] and keratinocytes [33] as well as promoting epidermal proliferation [42] and MCP-1 and MIP-1α predominantly recruit macrophages [1, 9]. Recent studies have shown that chemokines such as IL-8, MCP-1 and MIP-1α also contribute to angiogenesis [2, 19, 23, 25].

Cytokines and chemokines are known to be present in normal skin specimens as well as in wounded skin [22, 29, 40] and in the present study normal skin specimens also showed positive reactions for these chemokines (IL-8, MCP-1 and MIP-1α) in the epidermal cells and sweat gland cells. It is known that cytokines and chemokines are expressed in normal organs, which in principle, indicates the physiological significance of cytokines and chemokines for maintaining biological homeostasis [24]. Based on this consideration, the presence of chemokines in normal skin can be considered important for maintaining skin equilibrium, since the skin always receives various external stimuli and needs a relatively rapid turnover of cells.

For cellular sources of IL-8, MCP-1 and MIP-1α in the skin wound healing process [1, 12, 17, 18], it has already been reported that they are produced by neutrophils, macrophages, fibroblasts, endothelial cells and keratinocytes, and the immunohistochemical results in the present study confirmed these previous findings. Interestingly, the chemokines were localised in their target cells, thus indicating that these chemokines were expressed in the neutrophils and macrophages, respectively. This may suggest that neutrophils and macrophages are activated in an autrocrine manner.

According to previous studies [10, 12, 17, 20, 22, 39] the expression of chemokines such as IL-8, MCP-1 and

MIP-1α peaked within a few days after injury. Through morphometrical analysis in the present study, wound specimens with ages ranging between 1 and 4 days corresponding to the inflammatory phase, showed the highest mean positive ratio for these chemokines. This finding reconfirmed that chemokines such as IL-8, MCP-1 and MIP-1α play an indispensable biological role in the inflammatory phase of wound healing, especially in the recruitment of inflammatory leukocytes.

From the viewpoint of a forensic pathology application, the present study showed that IL-8, MCP-1 and MIP-1α are available as markers of wound age determination. In particular, IL-8 was considered to be the most useful among these three chemokines, since the most significant differences were found in the IL-8 positive ratio among the four groups. Therefore, ratios of > 50% for IL-8 indicate a wound age of at least 1 day. Although the wide variation gave no significant difference between MCP-1 and MIP-1α in groups II and III, the ratios of > 30% for MCP-1 or > 40% for MIP-1α could be detected in skin wounds with a wound age of at least 1 day, as well. Moreover, it is considered that the combined evaluation of these three chemokines can achieve a wound age determination with a high degree of accuracy and objectivity.

Acknowledgements The authors sincerely thank Mr. S. Sakai (Undergraduate student, Kanazawa University Faculty of Medicine) for his technical assistance. This study was financially supported by Grants-in-Aid for Scientific Research (T.O. No. 08457146) and Encouragement of Young Scientists (T.K. No. 10770187) from the Ministry of Education, Science, Sports and Culture of Japan as well as by the Grant-in-Aid of the Japanese Medical Association (T.O.).

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