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ORIGINAL ARTICLE

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The dynamics of inflammatory cytokines in the healing process of mouse skin wound: a preliminary study for possible wound age determination

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Abstract The dynamics of inflammatory cytokines such as interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNFα) during the healing process of mouse skin wounds were examined using the enzyme-linked immunosorbent assay and immunostaining. The applicability of this examination for wound age estimation is discussed from the perspective of forensic pathology. After wound induction, mice were sacrificed at intervals ranging from 0 to 240 h. The levels of TNFα and IL-1β began to elevate rapidly after wounding and reached a peak at 3 h. The IL-1α level reached a peak at 6 h, and IL-6 peaked at 12 h. An infiltration of numerous leukocytes, indicating *acute* inflammation, was observed at 3 and 6 h, and the main source of the cytokines was immunohistochemically identified as neutrophils. These results indicate that TNFα and IL-1β play an important role in the commencement of inflammation. "Rebound" of cytokine levels, i.e. a re-increase, was observed at 72 h after wounding. Histological examination of the 72-h-old wound showed migration of fibroblasts and the formation of new granulation tissues, indicating the proliferative stage of the wound healing process. These experimental findings indicate that these cytokines have a close relationship to wound remodeling as well as to inflammation. From the viewpoint of forensic pathology, it is considered that inflammatory cytokines may become one of the markers for wound age estimation, but further studies are needed, especially those involving the investigation using human wound specimens with known time intervals after injury.

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Introduction

Wound healing is a complex yet orderly phenomenon. There have been numerous previous studies on wound age estimation using temporal characteristics in the wound healing process (Oehmichen 1990). One of the most well recognized conventional histological methods, Berlin blue staining for phagocytosed hemosiderin granules by macrophages, has been routinely carried out in forensic practice. Raekallio (1972, 1976) reported the applicability of the histochemical measurement of several enzyme levels at the wound margin in estimating skin wound ages. Following the introduction of immunohistochemical techniques, it was systematically shown that extracellular matrix components such as collagen (an extracellular component of granulation tissue) and fibronectin (an extracellular adhesive glycoprotein) were useful markers (Eisenmenger et al. 1988; Betz et al. 1992a,b, 1993a,b; Betz 1994). In addition, the proliferative activity of different cell types can be examined using markers such as Ki-67 or bromodeoxyuridine (BrdU), and these results have been applied to the wound age estimation or determination of wound vitality (Betz et al. 1993c; Oehmichen and Cröpelin 1995).

At present, it is acknowledged that cytokines and growth factors, as biological mediators, contribute to the repair of the wound site (Fahey et al. 1990; Kurita et al. 1992; Bettinger et al. 1994). Biomedical studies on cytokines have been widely performed from both basic and practical viewpoints, and several cytokines have been found to play key roles in the inflammatory reaction (Baumann and Gauldie 1994). In particular, interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNFα) are very important chemical mediators in the *acute* inflammatory phase of wound healing (Wahl and Wahl 1992).

We have already examined the immunohistochemical localization of IL-1α by comparison to extracellular ma-

trix components and have discussed elsewhere whether the analysis of IL-1α localization with the passage of time may provide further information on the wound age estimation (Kondo and Ohshima 1995). In the present study, the temporal local activity of inflammatory cytokines during the healing process of mouse skin wound was examined, and the suitability of this procedure for wound age estimation is discussed from the perspective of forensic pathology.

Materials and methods

Eight-week-old male Crj-CD1 (ICR) mice, weight 30–35 g (Charles River Breeding Laboratories, Japan), were used as the experimental animals. They were deeply anesthetized by intra-peritoneal injection of pentobarbital sodium (5 μg/g), and a 1-cm-long incision was made with a scalpel in the skin layer in each dorsum. After wounding, each mouse was individually housed in a sterilized cage and given sterilized food as well as redistilled water in order to prevent bacterial infection. Ten mice were then sacrificed at each of the following times after wounding: 0, 10 and 30 min, and 1, 3, 6, 12, 24, 72, 144 and 240 h. The hairs of the dorsum were shaved, and each skin wound was examined using a surgical operating microscope. The wounded skin, including an intact 1-cm margin, was excised. As controls, the skin specimens of ten unwounded mice were also examined.

Each wound site or normal mouse skin was fixed in 10% formaldehyde with phosphate-buffered saline (PBS; pH 7.2). The tissue specimens were embedded in paraffin and sectioned at a thickness of 4 μm. Immunostaining for IL-1α, IL-1β, IL-6 and TNFα was performed using the appropriate primary antibody as shown in Table 1. Each primary antibody was reacted overnight at 4°C in a humid chamber. Biotinylated anti-rabbit IgG goat or anti-hamster IgG goat antibody was used as the secondary antibody and reacted at room temperature for 30 min. Thereafter, avidin-biotinylated peroxidase complex (ABC) method was conventionally employed. In addition, H-E staining was also performed.

Each wound site or normal mouse skin (ca. 0.09–0.14 g) was excised and then homogenized with 0.5 ml PBS (pH 7.2). The homogenized sample was centrifuged at 12000 rpm for 20 min at 4°C, and the supernatant was collected for quantitation of cytokines and total protein. Commercial enzyme-linked immunosorbent assay (ELISA) kits for the quantitation of IL-1α, IL-1β, IL-6 and TNFα were employed (IL-1α, IL-1β and IL-6 ELISA kits, Genzyme, USA; TNFα ELISA kit, Endogen, USA). All ELISA kits were used according to the instruction manuals. The lower detection limits of each ELISA kit were 15 pg/ml for IL-1α, 10 pg/ml for IL-1β, 5 pg/ml for IL-6, and 10 pg/ml for TNFα.

The total protein level was measured by Lowry's method (Lowry et al. 1951), and a calibration curve was prepared at concentrations of 0, 20, 40, 60, and 80 μg/ml using bovine serum albumin.

The cytokine level was evaluated using the following formula: cytokine level = cytokine (pg/ml)/total protein (μg/ml)/skin weight (g)

Statistical analysis was performed using the Smirnov test for extreme values.

Table 1 Primary antibodies used in immunohistochemical examination

Antigen	Clone	Immunized animal	Supplier
IL-1α	Polyclonal	Rabbit	Genzyme (USA)
IL-1β	Monoclonal	Hamster	Genzyme (USA)
IL-6	Monoclonal	Rat	Genzyme (USA)
TNFα	Polyclonal	Rabbit	Genzyme (USA)

All of the animal experiments in the present study were performed according to the Guidelines for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University.

Results

Normal skin

Keratinocytes showed a positive immunohistochemical reaction for all cytokines, although the degree of the positive reaction for IL-1β was lower than that of other cytokines. Collagen fibers were not immunostained with any antibody. The basal level of each cytokine, was detectable even in normal skin (Table 2).

After wounding (0–24 h)

Using a surgical operating microscope, acute inflammatory reactions were observed as a slight redness at the

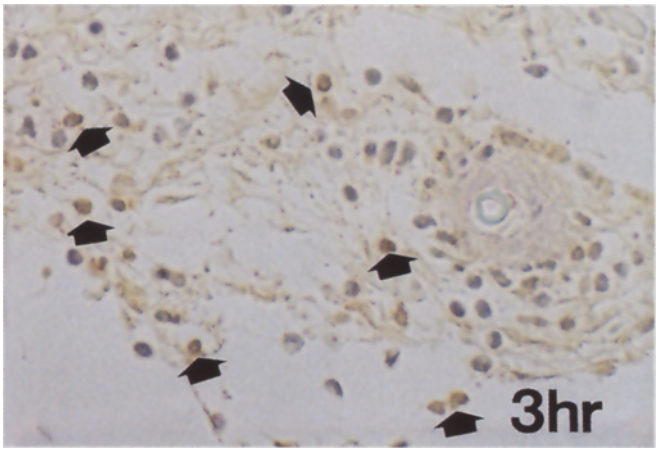

Fig. 1 A 3-h-old skin wound with infiltration of many neutrophils and positive immunoreaction for IL-1β (*arrows*; ABC method, × 400)

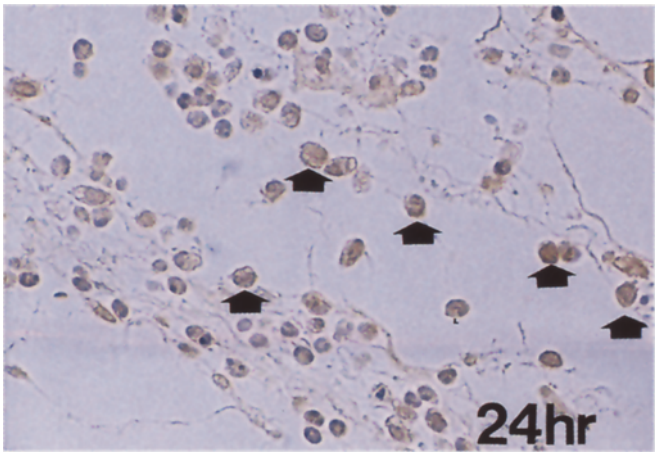

Fig. 2 In this 24-h-old skin wound, most of the neutrophils have disappeared at the wound site and have been replaced by phagocytic macrophages that show positive reactions for TNFα (*arrows*; ABC method, × 400)

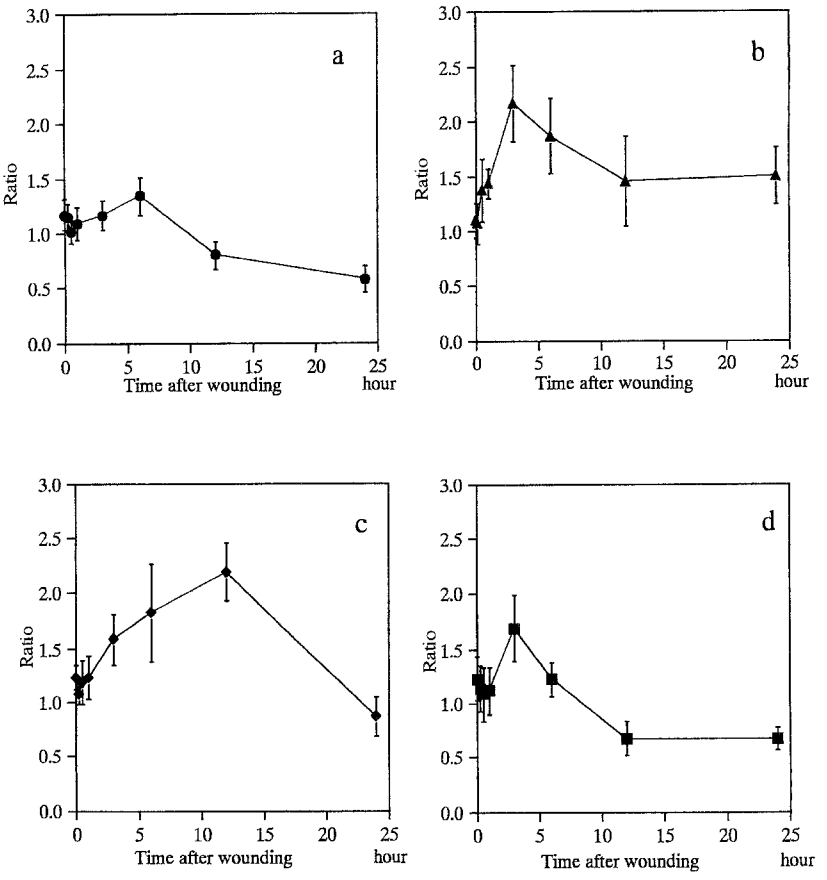

Fig. 3a–d The ratio of each cytokine level to the normal level 0–24 h after wounding. The data shown are the mean values ± SE for at least six mice per group at each time after wounding. **a** IL-1α; **b** IL-1β; **c** IL-6; **d** TNFα

wound margin of 3-h-old wounds, which became more clearly defined in 6-h-old wounds. At 24 h after wounding, inflammation was still observed around the wound margin.

Histologically, infiltration of numerous neutrophils, showing positive reactions for IL-1α, IL-1β, IL-6 and TNFα, was observed at 3- or 6-h-old wound sites (Fig. 1). At 24 h after wounding, neutrophils had largely disappeared from the wound site and had been replaced by phagocytic macrophages which showed positive reactions for IL-1α, IL-1β, IL-6 and TNFα in the cytoplasm (Fig. 2).

The levels of TNFα and IL-1β began to rapidly elevate soon after wounding and peaked at 3 h after wounding (Fig. 3b, d).Although the level of IL-1α showed a peak at 6 h after wounding, the increased ratio of IL-1α to normal skin was smaller than those of IL-1β, IL-6 and TNFα (Fig. 3a). At 12 h after wounding, the IL-6 level reached a peak, later than any other cytokine, and the increased ratio of IL-6 was almost as large as that of IL-1β (Fig. 3c).

After wounding (72–240 h)

During this stage, crust formation was noted, and skin redness around the wound margin had almost disappeared. At 240 h after wounding, the wound site was almost healed. Histological examination of the 72-h-old wound disclosed the migration of fibroblasts and the new formation of granulation tissues, indicating the proliferative stage of the wound healing process. Phagocytic macrophages and fibroblasts were also immunostained with anti-IL-1α,-IL-1β, -IL-6 and -TNFα antibodies (Fig. 4). Although each cytokine level decreased after the peak time, "rebound" of every cytokine level, i.e. a re-increase, was observed at 72 h after wounding (Fig. 5).

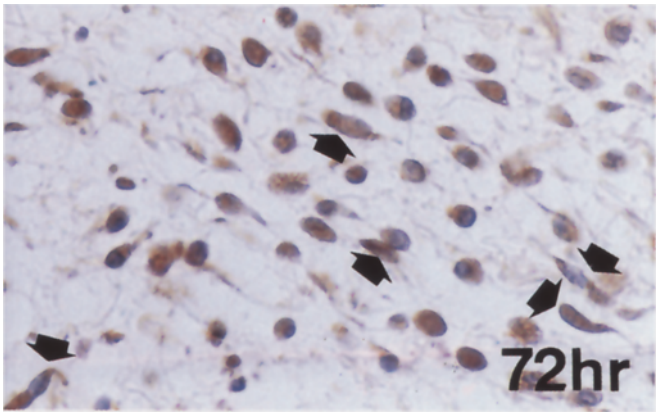

Fig. 4 A 72-h-old skin wound with positive staining for IL-6 found in proliferative fibroblasts as well as in macrophages (*arrows*; ABC method, × 400)

Discussion

IL-1 is a polypeptide that is produced by macrophages, fibroblasts and neutrophils as a response to invasive effects such as infection, injury or antigenic challenges (Oppen-

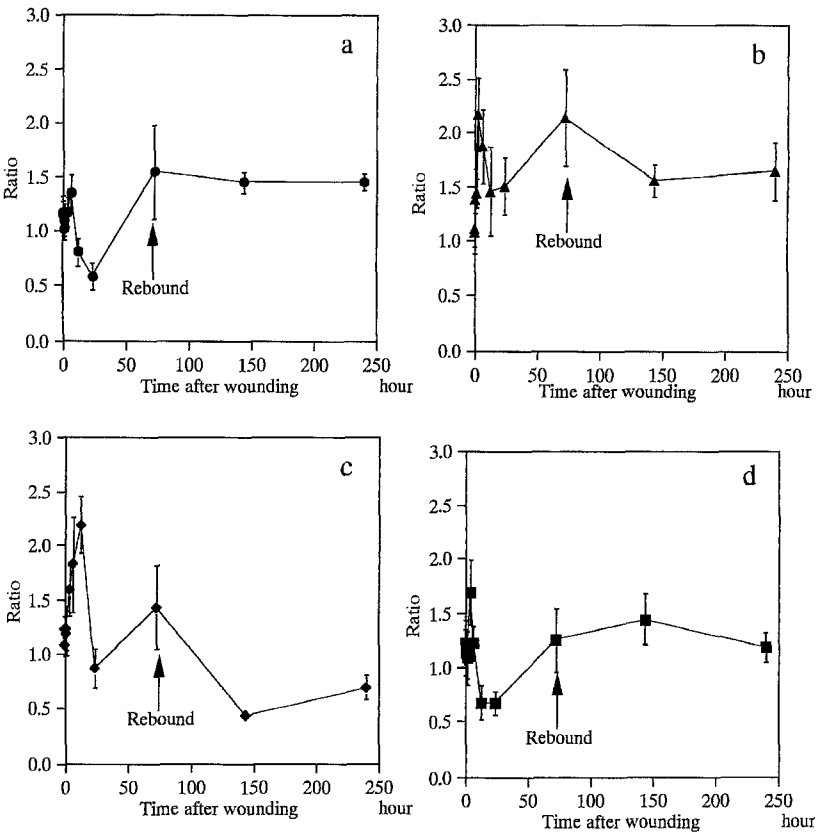

Fig. 5a–d The ratio of each cytokine level to the normal level 0–240 h after wounding. The data shown are the mean values ± SE for at least six mice per group at each time after wounding. At 72 h after wounding, rebound is observed for every cytokine level. **a** IL-1α; **b** IL-1β; **c** IL-6; **d** TNFα

Table 2 Cytokine levels of normal skin (pg/μg protein/g skin, mean ± standard error)

Cytokine	Level
IL-1α	27.0 ± 1.66
IL-1β	0.752 ± 0.086
IL-6	18.6 ± 1.78
TNFα	1.94 ± 0.338

heim et al. 1986). There are two distinct genes for IL-1 proteins(IL-1α and IL-1β) (Auron et al. 1984; Lomedico et al. 1984). Mature IL-1α and IL-1β proteins have different amino acid sequences, sharing only 26% similarity in human IL-1 (Dinarello 1994). However, there is no significant difference in biological effect between IL-1α and IL-1β, and IL-1 has the following biological effects: activation of neutrophils and fibroblasts,with production of fever, acute phase protein and bone resorption (Nishihara et al. 1989; Dinarello 1994). IL-1 has also several endocrinological effects (Besedovsky et al. 1986; Del Rey et al. 1987) and in addition induces the production of other cytokines in the acute inflammatory stage (Dinarello 1994).

TNFα is one of the macrophage-derived cytokines produced by neutrophils, astrocytes, Kupffer's stellate cells etc. (Tracey 1994) and can induce IL-1 production and afterwards act synergistically with IL-1 (Elias et al. 1987). These bioactivities induced by TNFα have been observed to overlap with those of IL-1 (Le and Vilček 1987; Wahl and Wahl 1992).

IL-6 is also a multifunctional cytokine produced by polymorphonuclear leukocytes, macrophages, fibroblasts, lymphocytes and epithelial cells, showing local and systemic effects during immunological and inflammatory reactions (Hirano 1992). Recent reports indicate that IL-6 is locally present in wound fluid from mice (Ford et al. 1989; Fahey et al. 1991) and has numerous biological activities, including B-cell, T-cell, and macrophage differentiation and stimulation of keratinocyte growth that may impact on wound healing (Hirano 1992). On the other hand, IL-6 has been implicated in systemic effects such as the induction of acute phase protein synthesis, the stimulation of hematopoiesis, and the release of adrenocorticotrophic hormone after injury (Hirano 1992). Furthermore, IL-1 induces IL-6 production, and IL-1 and IL-6 synergistically induce T-cell proliferation and the release of acute phase proteins.

It is known that wound healing in mammalian species, including mice, rats, guinea pigs and rabbits, is very similar to human wound healing, although there is an anatomical difference between human skin and mouse skin in that mouse skin contains a subcutaneous panniculus carnosus muscle. We used mice as a model of human wound healing, since mice are often used as a model to investigate the dynamics of cytokines during wound healing (Ford et al. 1989; Fahey et al. 1991).

Our results (Table 2) demonstrate that the inflammatory cytokines examined in the present study are present at basal values in normal skin. These results can be presumed to show that production of these cytokines is also induced in the skin, since the skin is always stimulated from the external environment.

After bacterial infection or trauma, IL-1 and TNFα are initially produced by activated macrophages and appear

to be uniquely important in initiating the next series of reactions. IL-1 and TNFα are, therefore, known as "alarm cytokines" (Baumann and Gauldie 1994). Although IL-1α and IL-1β have similar biological properties, the production of much more IL-1β than IL-1α is induced in the acute inflammatory phase after bacterial infection or major trauma. In the present study, IL-1β and TNFα peaked earlier than other cytokines at 3 h, and the increase in IL-1β or TNFα relative to normal skin was larger than that in IL-1α. TNFα and IL-1β thus play important roles in the initiation of the inflammation response.

There are a few experimental studies on IL-6 activity using polyvinyl alcohol sponges implanted in mice. Ford et al. (1989) and Fahey et al. (1991) reported that the IL-6 level in the wound fluid reached a peak at 1 or 3 days after wounding. However, Mateo et al. (1994) showed that IL-6 activity in the wound fluid peaked at 12 h after wounding. Our results showed good agreement with those of Mateo et al., and, in the present, study IL-6 peaked later than other cytokines. The delay of the IL-6 peak can be explained by the postulated sequence in which IL-6 production is induced by IL-1.

At 72 h after wounding, a rebound of each cytokine level was immunochemically observed. Histologically, fibroblast infiltration and newly formed granulation tissue were seen in the wound site, and the sources of these cytokines were immunohistochemically identified as fibroblasts and macrophages. Thus, these inflammatory cytokines seem to have a close relationship with wound remodeling as well as inflammation.

Our results demonstrate not only that these inflammatory cytokines play important roles as chemical mediators in wound healing as a local vital reaction but also that they may become markers for wound age estimation. However, these results can not be directly applied to human skin wound age estimation in practical forensic cases, since they were obtained in animal experiments carried out under rigorously controlled conditions. The conditioned animal experiments in the present study did not take account of such variations as the wound size, depth of wounding, type of wound, or post-mortem interval, for reasons of the simplification of experiments. Therefore, further studies are required, especially through the investigation using the human wound specimens with known time interval after injury, with due consideration of post-mortem changes and the differences among individuals.

From our results, the level or cytological localization of only a single inflammatory cytokine does not necessarily provide enough information for wound age estimation. Therefore, it is indispensable to examine at least several kinds of cytokines and to comprehensively assess macroscopic findings and conventional histological findings (e.g. H&E staining) as well as immuno-histochemical or -chemical findings. If possible, other markers such as those of the extracellular matrix (collagen, fibronectin, etc.) should also be examined.

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