Int J Legal Med (2000) 113 : 140–145

ORIGINAL ARTICLE

Y. Sato · T. Ohshima

The expression of mRNA of proinflammatory cytokines during skin wound healing in mice: a preliminary study for forensic wound age estimation (II)

Received: 16 February 1999 / Received in revised form: 9 April 1999

Abstract This study examined the temporal expression of mRNA for the proinflammatory cytokines, interleukin (IL)-1α, IL-1β, IL-6 and tumor necrosis factor-α (TNFα) in incised wounds in mice using the reverse transcriptase-polymerase chain reaction (RT-PCR) and in situ hybridization (ISH) techniques. After incision, an increase in each cytokine mRNA level was observed by RT-PCR. The local IL-6 mRNA level peaked at 6 h, while the peak levels of mRNA for IL-1α, IL-1β and TNFα occurred between 48 and 72 h. All cytokine mRNA levels were almost normalized after 240 h. In the early phase of wound healing, infiltrating polymorphonuclear cells were labeled with antisense probes for IL-1α, IL-1β and TNFα mRNA by the ISH technique. Thereafter, infiltrating mononuclear cells and spindle-shaped mesenchymal cells showed positive signals for all the cytokines examined. Regenerating epidermal cells were also labeled with the antisense probes for IL-1α, IL-6 and TNFα mRNA, indicating that IL-1, IL-6, and TNFα are involved in skin wound healing and their local production by various cells involved in the healing process is suggested. From the viewpoint of forensic pathology, the temporal characteristics of the cytokine mRNA expression may have a potential to indicate wound age or wound vitality.

Key words Wound healing · Wound age estimation · Skin · Proinflammatory cytokine · Messenger RNA

This study was presented in part at the 3rd World Congress on Inflammation, Tokyo, November 1997

Y. Sato (✉) · T. Ohshima
Department of Legal Medicine,
Kanazawa University Faculty of Medicine, School of Medicine,
Takara-machi 13–1, Kanazawa 920–8640, Japan
Tel. +81-76-265-2222, Fax +81-76-234-4234

Introduction

Wound healing requires a complex interaction of different cell types and regulation by various cytokines, growth factors and other bioactive molecules. Proinflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor-α (TNFα) mediate the cellular responses that are critical to wound healing. Using a mouse model for healing of incised wounds, we previously examined the temporal expression of IL-1α, IL-1β, IL-6 and TNFα proteins at the wound site and demonstrated that these cytokines could be markers for wound age estimation [1]. In fact, immunohistochemical localization of IL-1α could be a possible indicator of wound age in human skin wounds [2].

Recently, we suggested the availability of mRNA analysis for forensic wound examination by the use of the reverse transcriptase-polymerase chain reaction (RT-PCR), because a semi-quantitative detection of IL-10 mRNA was possible from injured skin specimens within the postmortem interval of 5 days [3]. Based on the results of our previous studies, the temporal characteristics of mRNA expression of the proinflammatory cytokines may be applicable to practical forensic cases as an indicator of wound age or wound vitality. Prior to such an approach, basic experimental data on the temporal expression of the cytokine mRNA during skin wound healing are necessary.

This study examined the expression of IL-1α, IL-1β, IL-6 and TNFα mRNA at the incised wound site in mice over a 10-day time period using RT-PCR and in situ hybridization (ISH) techniques.

Material and methods

Preparation of wound tissue

A total of 45 male 8-week-old Crj-CD1 (ICR) mice, weighing 30–37 g (Charles River Breeding Laboratories, Japan), were anesthetized with an intra-peritoneal administration of sodium pentobarbital (5 μg/g). After shaving the dorsal region, a 2 cm full-thickness incision was made on the dorsal skin using a scalpel. Thereafter, the mice were fed sterilized food and redistilled water. At 1, 3, 6, 12, 24, 48, 72, 144 and 240 h following the incision, a total of 5 mice were sac-

Table 1 Sequences of the primers used for PCR and nested-PCR

Molecule		Sequence (5′ to 3′)
IL-1α	sense	TGGCCAAAGTTCCTGACTTGTTTG
	antisense	CAGGTCATTTAACCAAGTGGTGCT
	sense*	TTCAAGGAGAGCCGGGTGACAG
	antisense**	GGTAGGTGTAAGGTGCTGATCTGG
IL-1β	sense	ATGGCAACTGTTCCTGAACTCAACT
	antisense	CAGGACAGGTATAGATTCTTTCCTTT
	sense*	CGGACCCCAAAAGATGAAGG
	antisense**	GGTATTTTGTCGTTGCTTGGTTCTCC
IL-6	sense	CGTGGAAATGAGAAAAGAGTTGTGC
	antisense	ATGCTTAGGCATAACGCACTAGGTT
	sense*	AGCCAGAGTCCTTCAGAGAGATACAG
	antisense**	GGTCCTTAGCCACTCCTTCTG
TNFα	sense	CAGCCTCTTCTCATTCCTGCTTGTG
	antisense	CTGGAAGACTCCTCCCAGGTATAT
	sense*	GGCCTCCCTCTCATCAGTTC
	antisense**	TTGACGGCAGAGAGGAGGTTGACT
β-actin	sense	TTCTACAATGAGCTGCGTGTGGC
	antisense	CTCATAGCTCTTCTCCAGGGAGGA

*,**; Primers used for the nested-PCR to prepare RNA probes with the addition of T7(*)- and Sp6(**)-RNA polymerase promoter sequences to the 5′ end

rificed by cervical dislocation. A 2 × 1 cm area surrounding the wound was excised and the skin specimens were frozen in liquid nitrogen. One half of the specimen was used for RT-PCR, while the other half was used for ISH. As a control, skin specimens from five mice without incision were examined. Animals were cared for in accordance with the Guidelines for the Care and Use of Laboratory Animals of Takara-machi Campus of Kanazawa University.

Semi-quantitative RT-PCR

Semi-quantitation of mRNA using RT-PCR was performed as previously described [3]. Briefly, total RNA was isolated from skin specimens (weight 50 mg) using the RNA extraction kit ISOGEN (Nippon Gene, Japan). The cDNA was synthesized according to the standard protocol of the First-Strand cDNA Synthesis Kit (Life Science, USA). The resulting cDNA was used for PCR with the sequence-specific primer pairs listed in Table 1. The amplification, consisting of denaturation at 94 °C for 1 min, annealing at 65 °C for 2 min and extension at 72 °C for 1 min, was performed for 30 cycles in separate tubes for each primer pair. An experiment on cycle number titration revealed that the amplification reaction did not plateau within the cycles used for any primer pair (results not shown). Aliquots of the PCR products were subjected to 2% agarose gel electrophoresis and stained with ethidium bromide. The PCR products were visualized under UV light and photographed which were scanned into a computer and the luminescence of ethidium bromide staining for the PCR products was determined using the Macintosh densitometry program NIH image. Because β-actin mRNA is considered to be expressed at a constant amount in the cell, the ratio between determined value for the cytokines and β-actin was calculated to compare the samples of skin specimens obtained 1–240 h after incision.

ISH technique

Preparation of tissue sections

Serial 10 μm frozen sections were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 min and incubated at 37 °C for 10 min with 2.0 μg/ml proteinase K in 10 mM Tris-HCl and 1 mM EDTA. After postfixation for 10 min in 4% PFA in PBS, the slides were treated with 0.2 N HCl for 10 min, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, dehydrated in graded concentrations of ethanol and air-dried.

Preparation of RNA probe

To increase the binding specificity of the probes to complementary sequences, nested-PCR was performed using 1 μL aliquots of the RT-PCR product as template DNA and the primers listed in Table 1. The resulting PCR products containing T7- and Sp6-RNA polymerase promoter were used to prepare digoxigenin (DIG)-labeled RNA probes. The complementary (antisense) and anticomplementary (sense) probes corresponding to gene transcripts were obtained by in vitro transcription (DIG RNA Labeling Kit, Boehringer Mannheim Biochemica, Germany) according to the manufacturer's instructions. The single-strand RNA probes were washed by ethanol precipitation and resuspended in diethylpyrocarbonate-treated dH_2O. The sense probes were used as negative controls.

Hybridization and immunodetection of ISH signals

The sections were incubated for 1 h at 37 °C with a prehybridization mixture containing 50% deionized formamide, 10 mM Tris-HCl pH 7.6, 10% dextran sulfate, 1 × Denhardt's solution, 200 μg/ml yeast transfer RNA and 250 μg/ml salmon sperm DNA. After adding the RNA probes to the prehybridization mixture (approximately 200 ng/ml), hybridization was performed for 16 h at 50 °C in a moist chamber. The slides were subsequently washed in 2 × sodium chloride-sodium citrate solution (SSC) containing 50% formamide for 20 min at 50 °C, twice in 2 × SSC for 30 min at 50 °C and in 0.1 × SSC for 30 min at 50 °C. After washing with DIG-buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl) and blocking with 1.5% blocking reagent in DIG-buffer 1 for 1 h, the slides were incubated with a 1:500 dilution of alkaline phosphatase-conjugated anti-DIG polyclonal antibody (Boehringer Mannheim Biochemica) for 1 h at room temperature. The sections were washed twice in DIG-buffer 1 for 15 min each time, equilibrated with DIG-buffer 3 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 3 min and incubated with a color-substrate solution containing nitroblue tetrazolium salt (450 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (175 μg/ml) in DIG-buffer 3 in a darkroom.

Results

Temporal expression of cytokine mRNA

Each cytokine mRNA was detected in skin specimens by RT-PCR throughout the 10 days after incision (Fig. 1). After incision, there was an increase in each cytokine mRNA level. Comparison of the mRNA expression of the cytokines with that of β-actin mRNA showed that the peak level for IL-6 mRNA occurred at 6 h, while mRNA levels for IL-1α, IL-1β and TNFα peaked between 48 and 72 h (Figs. 2 & 3). The ratio of increase in IL-1β and IL-6 compared to the normal level appeared to be larger than those of other cytokines. All of the cytokine mRNA levels decreased after the peak and were almost normalized at 240 h.

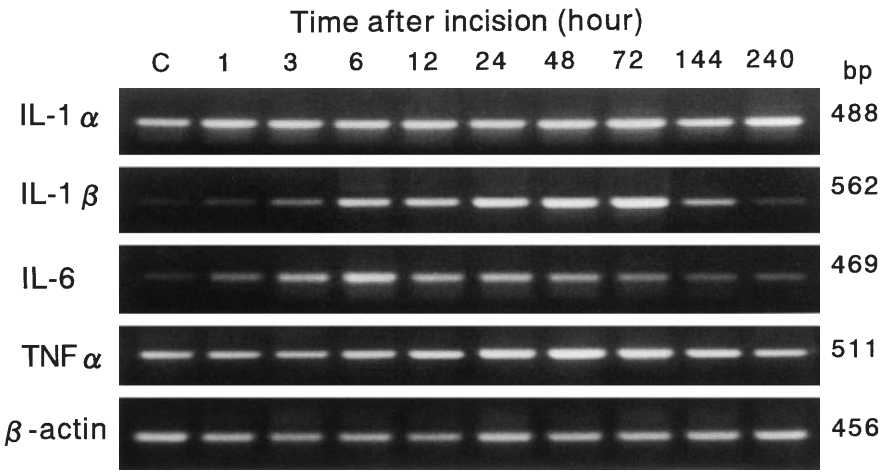

Fig. 1 RT-PCR analysis of mRNA expression of proinflammatory cytokines during skin wound healing in mice. The size in base pairs of the amplified cDNA fragments is indicated. Lane C indicates the results of normal mice (control)

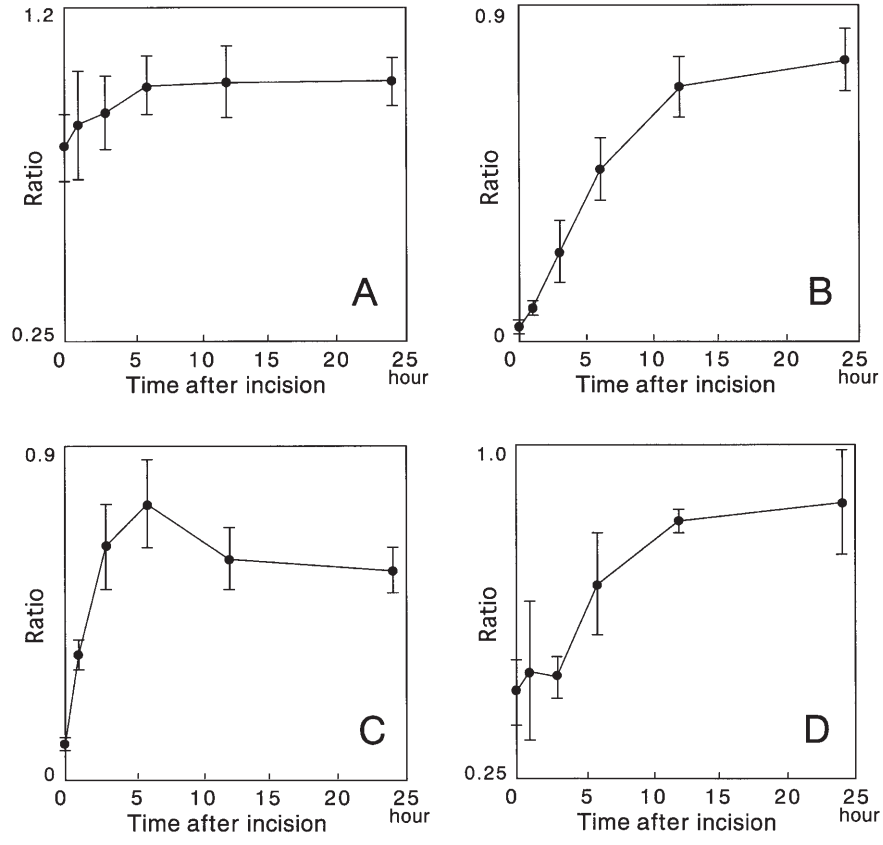

Fig. 2 A–D Temporal expression of mRNA for proinflammatory cytokines 1–24 h after incision. The results represent the mean ± SEM of 5 mice per group. The data of 0 h after incision represent the results obtained from normal mice: A IL-1α; B IL-1β; C IL-6; D TNFα

Cellular sources of cytokine mRNA

Positive hybridization signals of the antisense probes for IL-1α, IL-6 and TNFα mRNA were detected in epidermal cells of normal skin, but cells positive for IL-1β mRNA were not detected in the dermis or epidermis (not shown). When the sense probes for cytokine mRNA were used, no signals other than background staining were observed (not shown), suggesting the specificity of positive signals for target sequences. These positive hybridization signals were continuously present in the epidermal layer of the skin during the healing process.

A strong infiltration of polymorphonuclear cells (PMNs) was evident between 6 and 12 h and these cells were labeled with the antisense probes for IL-1α, IL-1β and TNFα (Fig. 4A), but the PMNs lacked positive signals for IL-6. The nuclei appeared stained in some cells, but this phenomenon was interpreted as originating from the overlying cytoplasm rather than intrinsic nuclear staining.

Within 48–72 h, the PMNs had been largely replaced by mononuclear cells (MNCs) and granulation tissue had invaded the incision space. In the cytoplasm of the MNCs, positive signals for all of the cytokines examined were detected (Fig. 4B).

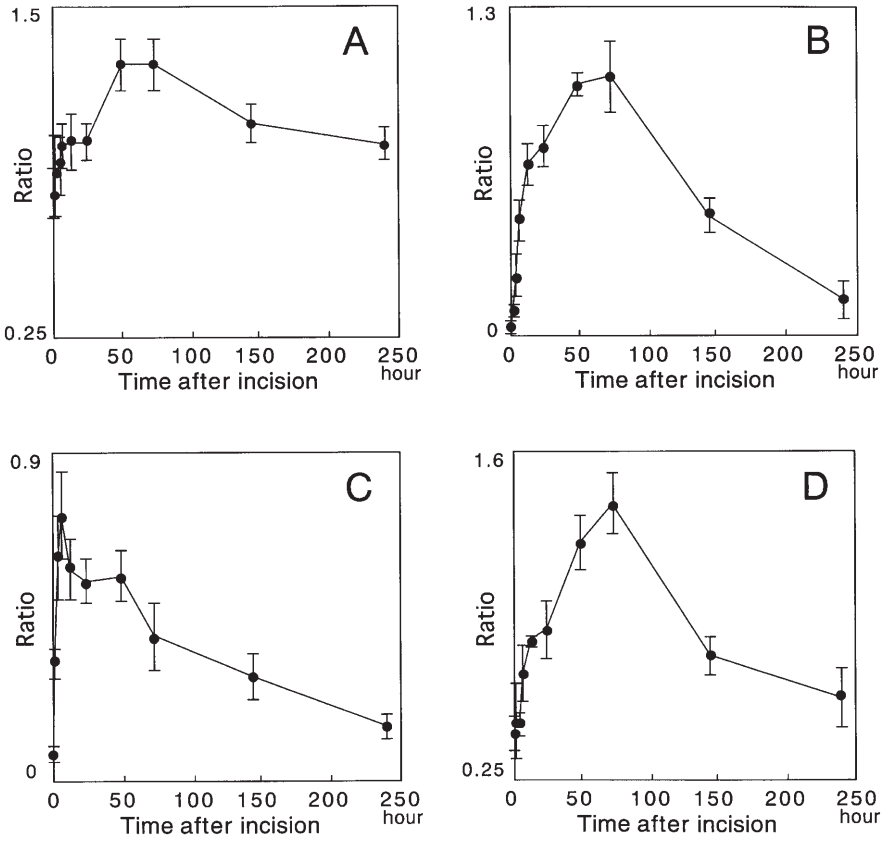

Fig. 3 A–D Temporal expression of mRNA for proinflammatory cytokines 1–240 h after incision. The results represent the mean ± SEM of 5 mice per group. The data of 0 h after incision represent the results obtained from normal mice: A IL-1α; B IL-1β; C IL-6; D TNFα

Reepithelialization was evident at 144 h, yielding a thickened epidermal layer. The regenerating epidermal cells showed positive signals for IL-1α, IL-6 and TNFα (Fig. 4C), but not for IL-1β. In addition to the MNCs, localization of all of the cytokine mRNA signals was observed in spindle-shaped mesenchymal cells (Fig. 4D). The wounds were almost healed at 240 h after incision.

Discussion

In normal skin, mRNA for each proinflammatory cytokine was detected by RT-PCR and the epidermal cells were labeled with the antisense probes for IL-1α, IL-6 and TNFα. The absence of IL-1β mRNA-positive signals in the epidermal cells was in agreement with previously published results [4]. The expression of the cytokines under normal conditions may be related to their role in the normal homeostasis of skin function [5].

IL-1 may be important for the wound healing process because it has various effects on cells involved in wound healing such as induction of inflammatory cell migration and stimulation of fibroblast proliferation [6]. Previous reports showed that there was increased production of IL-1 in injured skin specimens [7, 8] and that treatment with recombinant IL-1α enhanced wound reepithelialization [9]. The present study provided further evidence for the involvement of IL-1 in the healing process of mouse skin wounds.

Like IL-1, IL-6 has broad stimulatory properties as a major mediator of the acute-phase response [10]. In this study, the local IL-6 mRNA level increased more rapidly than the IL-1 and TNFα mRNA levels and peaked 6 h after incision. These results suggest that IL-6 may be required for controlling the acute inflammatory response [11]. Even though it is unclear whether IL-6 expression can be modulated in the PMNs [12], the peak level of IL-6 mRNA might be partially due to the increase in IL-6 mRNA induction by the epidermal cells, because the infiltrating PMNs lacked positive hybridization signals for IL-6.

TNFα shares many of the biological activities of IL-1 [13]. The temporal expression of TNFα mRNA showed a similar pattern to that of IL-1β mRNA, and the peak level was observed 3 days after incision. In situ accumulation of MNCs at the wound site also reached a peak at 3 days and these MNCs were labeled with the antisense probes for the cytokines. These findings indicate that the MNCs, probably macrophages, and their related products may play a critical role in the healing process [14].

According to our previous study [1], local protein levels of IL-1, IL-6 and TNFα peaked within 12 h after incision, decreased thereafter, and increased again to a peak between 3 and 6 days. IL-6 mRNA expression in the present study almost correlated with the temporal course of IL-6 protein level. However, no significant increase in the mRNA level corresponding to the early peak of IL-1 and TNFα protein levels could be found under the experimental conditions of this study. This discrepancy can be ex-

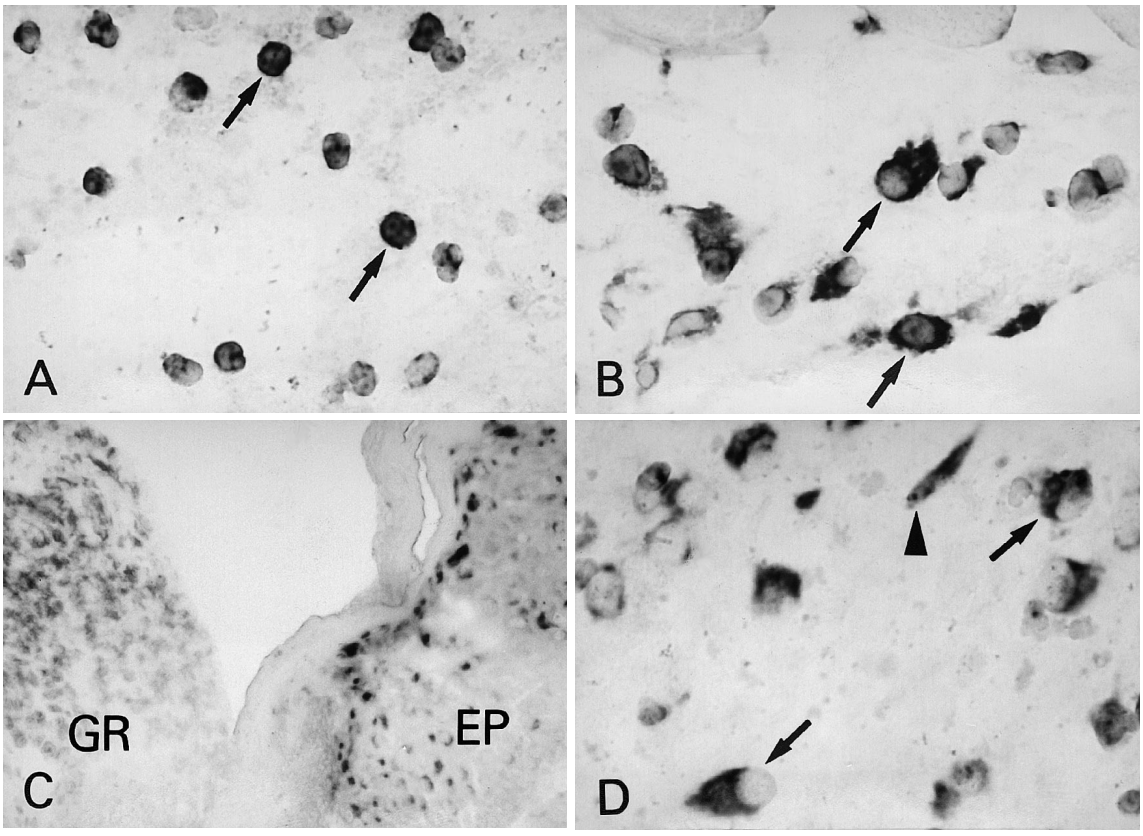

Fig. 4 A–D In situ hybridization of mRNA for proinflammatory cytokines during skin wound healing: A 12 h after incision with TNFα antisense probe. Arrows indicate polymorphonuclear cells, probably neutrophils; B 3 days after incision using IL-1β antisense probe. Arrows indicate relatively large mononuclear cells, probably macrophages; C 6 days after incision using IL-1α antisense probe. Positive signals were detected in the epidermal layer (EP) and the granulation tissue (GR); D 6 days after incision using IL-6 antisense probe. Arrows and arrowhead indicate mononuclear cells and spindle-shaped mesenchymal cell, probably macrophages and fibroblast, respectively (Original magnification; A 360, B 360, C 180, D 360)

plained in part as follows, although further experimental data should be obtained: after injury, the exudation of blood plasma occurs within 1 h, followed by migration of neutrophils toward the wound site. The exudate in the extravascular tissues (wound fluid) has been shown to contain IL-1 and TNFα [15]. Therefore, it may be considered that the early peak of IL-1 and TNFα protein levels is partially due to the exudated wound fluid as well as de novo synthesis of the proteins.

Most of the recent studies on wound age estimation used the technique of immunohistochemistry [2, 16–21]. The results of the immunostaining, however, must be carefully interpreted because of the influence of postmortem changes on immunohistochemical reactions [22] and postmortem induction of the proteins [23]. The techniques of molecular biology have been also applied to wound age estimation. Betz et al. [24] examined the time-dependent appearance of apoptotic cell death in human skin wounds and Oehmichen and Lagodka [25] investigated time-dependent RNA synthesis during skin wound healing in rats.

Recently, we demonstrated that increased expression of IL-10 mRNA could indicate wound vitality in injured skin specimens of mice and that β-actin mRNA was detectable by RT-PCR from skin specimens within a postmortem interval of 11 days [3]. Based on the results of our previous studies, the semi-quantitative detection of mRNA for the proinflammatory cytokines seems to be useful for wound age estimation. However, wound age estimation using this method must be considered with some concern, because we have noted that the IL-10 to β-actin ratio increased during the postmortem interval, which might reflect postmortem induction of IL-10 mRNA in cells after somatic death [3].

We consider that the increase in mRNA expression of the proinflammatory cytokines after injury has a potential to indicate wound vitality. IL-1β and IL-6 mRNA may be especially useful for this purpose, because the ratio of increase in IL-1β and IL-6 compared to the normal level appeared to be larger than those of other cytokines. Furthermore, the study using the ISH technique revealed that the increase in mRNA levels of the cytokines reflected local induction, i.e. a local vital reaction.

In conclusion, this study demonstrates that IL-1, IL-6, and TNFα are involved in skin wound healing and a local production by various cells involved in the healing process is suggested. From the viewpoint of forensic pathology, the temporal characteristics of the cytokine

mRNA expression may have a potential to indicate wound age or wound vitality, and therefore, further studies using human skin wounds with known postinfliction intervals are necessary.

Acknowledgements The authors would like to thank Prof. Dr. Yasuni Nakanuma and Dr. Ken-ichi Harada (Second Department of Pathology, Kanazawa University Faculty of Medicine, School of Medicine, Kanazawa, Japan) for their kind instruction in ISH technique. This study was financially supported by a Grant-in-Aid for General Scientific Research 08457146 (to TO) from the Ministry of Education, Science, Sports and Culture of Japan.

References

1. Kondo T, Ohshima T (1996) The dynamics of inflammatory cytokines in the healing process of mouse skin wound: a preliminary study for possible wound age determination. Int J Legal Med 108:231–236
2. Kondo T, Ohshima T, Eisenmenger W (1999) Immunohistochemical and morphometrical study on the temporal expression of interleukin-1α (IL-1α) in human skin wounds for forensic wound age determination. Int J Legal Med 112:249–252
3. Ohshima T, Sato Y (1998) Time-dependent expression of interleukin-10 (IL-10) mRNA during the early phase of skin wound healing as a possible indicator of wound vitality. Int J Legal Med 111:251–255
4. Takács L, Kovacs EJ, Smith MR, Young HA, Durum SK (1988) Detection of IL-1α and IL-1β gene expression by in situ hybridization: tissue localization of IL-1 mRNA in the normal C57BL/6 mouse. J Immunol 141:3081–3095
5. McKay IA, Leigh IM (1991) Epidermal cytokines and their roles in cutaneous wound healing. Br J Dermatol 124:513–518
6. Oppenheim JJ, Kovacs EJ, Matsushima K, Durum SK (1986) There is more than one interleukin 1. Immunol Today 7:45–56
7. Fahey TJ III, Sherry B, Tracey KJ, van Deventer S, Jones WG II, Minei JP, Morgello S, Shires GT, Cerami A (1990) Cytokine production in a model of wound healing: the appearance of MIP-1, MIP-2, cachectin/TNF and IL-1. Cytokine 2:92–99
8. Robertson FM, Pellegrini AE, Ross MS, Oberyszyn AS, Boros LG, Bijur GN, Sabourin CK, Oberyszyn TM (1995) Interleukin-1α gene expression during wound healing. Wound Rep Reg 3:473–484
9. Sauder DN, Kilian PL, McLane JA, Quick TW, Jakubovic H, Davis SC, Eaglstein WH, Mertz PM (1990) Interleukin-1 enhances epidermal wound healing. Lymphokine Res 9:465–473
10. Hirano T (1992) The biology of interleukin-6. Chem Immunol 51:153–180
11. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, Achong MK (1998) IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. J Clin Invest 101:311–320
12. Cassatella MA (1995) The production of cytokines by polymorphonuclear neutrophils. Immunol Today 16:21–26
13. Le J, Vilček J (1987) Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Lab Invest 56:234–248
14. Leibovich SJ, Ross R (1975) The role of the macrophage in wound repair: a study with hydrocortisone and antimacrophage serum. Am J Pathol 78:71–100
15. Ford HR, Hoffman RA, Wing EJ, Magee DM, McIntyre L, Simmons RL (1989) Characterization of wound cytokines in the sponge matrix model. Arch Surg 124:1422–1428
16. Betz P, Nerlich A, Wilske J, Tübel J, Wiest I, Penning R, Eisenmenger W (1992) Immunohistochemical localization of fibronectin as a tool for the age determination of human skin wounds. Int J Legal Med 105:21–26
17. Betz P, Nerlich A, Wilske J, Tübel J, Penning R, Eisenmenger W (1993) Analysis of the immunohistochemical localization of collagen type III and V for the time-estimation of human skin wounds. Int J Legal Med 105:329–332
18. Betz P (1995) Collagen subtypes-markers for the healing of skin wounds. In: Oehmichen M, Kirchner H (eds) Research in legal medicine, vol 13. The wound healing process: forensic pathological aspects. Schmidt-Römhild, Lübeck, pp 247–256
19. Dreßler J, Bachmann L, Kasper M, Hauck JG, Müller E (1997) Time dependence of the expression of ICAM-1 (CD 54) in human skin wounds. Int J Legal Med 110:299–304
20. Dreßler J, Bachmann L, Koch R, Müller E (1998) Enhanced expression of selectins in human skin wounds. Int J Legal Med 112:39–44
21. Hausmann R, Nerlich A, Betz P (1998) The time-related expression of p53 protein in human skin wounds-a quantitative immunohistochemical analysis. Int J Legal Med 111:169–172
22. Fieguth A, Kleemann WJ, von Wasielewski R, Werner M, Tröger HD (1997) Influence of postmortem changes on immunohistochemical reactions in skin. Int J Legal Med 110: 18–21
23. Grellner W, Dimmeler S, Madea B (1998) Immunohistochemical detection of fibronectin in postmortem incised wounds of porcine skin. Forensic Sci Int 97:109–116
24. Betz P, Nerlich A, Tübel J, Wiest I, Hausmann R (1997) Detection of cell death in human skin wounds of various ages by an in situ end labeling of nuclear DNA fragments. Int J Legal Med 110:240–243
25. Oehmichen M, Lagodka T (1991) Time-dependent RNA synthesis in different skin layers after wounding. Experimental investigations in vital and postmortem biopsies. Int J Legal Med 104:153–159