Int J Legal Med (1998) 111 : 251–255

ORIGINAL ARTICLE

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Time-dependent expression of interleukin-10 (IL-10) mRNA during the early phase of skin wound healing as a possible indicator of wound vitality

Received: 1 December 1997 / Received in revised form: 15 January 1998

Abstract This study was performed to clarify whether interleukin-10 messenger RNA (IL-10 mRNA) could be a possible indicator for the distinction between intravital wounds and postmortem damage. After incision, mice were sacrificed from 0 to 180 min. The initial amount of IL-10 mRNA in each skin specimen was evaluated using the reverse transcriptase-polymerase chain reaction (RT-PCR). After 15 min there was a rapid increase in IL-10 mRNA which peaked at 60 min. A significant increase in IL-10 mRNA occurred between 30 and 180 min. During the 5 day postmortem interval the increase in time-dependent IL-10 mRNA expression was maintained and no significant increase in IL-10 mRNA expression occurred in the postmortem control. The increased expression of IL-10 mRNA could be considered a vital reaction in skin specimens with postmortem change. This study demonstrated the possible use of mRNA analysis for forensic wound examination because mRNA was detectable by RT-PCR over a longer postmortem time course.

Key words Wound healing · Wound vitality · Interleukin-10 (IL-10) · Messenger RNA · Reverse transcriptase-polymerase chain reaction (RT-PCR)

Introduction

The wound healing process is characterized by acute inflammation, granulation tissue formation, matrix formation and remodeling. Various biological mediators such as cytokines and growth factors contribute to the healing process during the phases of wound healing. Interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNFα) are multifunctional cytokines necessary for healing during the acute inflammatory phase. Use of these inflammatory cytokines for forensic wound age estimation has been also investigated [1].

Interleukin-10 (IL-10) may contribute to the acute inflammatory phase of the healing process, because the biosynthesis of inflammatory cytokines by monocytes or macrophages is suppressed by the inhibitory cytokine IL-10 [2]. We previously quantified IL-10 protein during the healing process of mouse skin wounds using an enzyme-linked immunosorbent assay [3]. We demonstrated that the local IL-10 protein level rapidly increased after incision and peaked at 3 h. In those studies, IL-10 messenger RNA (mRNA) expression was not determined. If there is a significant increase in IL-10 mRNA after incision, IL-10 mRNA instead of IL-10 protein would be a more sensitive indicator of wound vitality, because the induction of IL-10 mRNA occurs before protein production. There is no previous study using mRNA analysis for forensic wound examination and the effect of postmortem changes on mRNA remains unknown.

This study determined whether IL-10 mRNA could indicate the difference between postmortem damage and intravitally sustained wounds, specifically for short post infliction intervals. Using a mouse model of skin wound healing after incision, the temporal expression of IL-10 mRNA at the wound site and the effect of postmortem changes on mRNA were investigated using a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Material and methods

Expression of IL-10 mRNA in situ

Animal protocol

A total of 30 male 8-week-old Crj-CD1 (ICR) mice, weighing 30–37 g (Charles River Breeding Laboratories, Japan), were anesthetized with an intra-peritoneal injection of sodium pentobarbital (5 μg/g). A 2 cm full thickness incision was performed on the dorsal skin of mice using a scalpel and the mice were returned to ster-

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ile cages and provided sterilized food and redistilled water. A total of 5 mice were sacrificed at 0, 5, 15, 30, 60 and 180 min post-incision. After shaving the dorsal region a 2 × 1 cm area of skin surrounding the wound was excised. As a control, skin specimens from 5 mice without incision were examined.

The animal experiments were performed under the Guidelines for the Care and Use of Laboratory Animals at Takara-machi Campus of Kanazawa University.

RT-PCR

The skin specimens (wet weight 50 mg) were placed in a sterile 1.5 ml-tube and total RNA was isolated using a modified acid guanidinium thiocyanate-phenol-chloroform method (ISOGEN RNA extraction kit, Nippon Gene, Japan) according to the manufacturer's instructions. The RNA pellet was air-dried for 10 min and resuspended in 20 µ1 diethylpyrocarbonate (DEPC)-treated dH_2O. Using 2 µl of the RNA samples, cDNA synthesis was performed in a 25 µl reaction mixture which contained 25 U of avian myeloblastosis virus reverse transcriptase (AMV-RT), 12.5 U of RNase inhibitor, 0.5 µg of oligo-$dT_{12\text{-}18}$ primers, 10 mM DTT and buffer provided by the manufacturer (First-strand cDNA synthesis kit, Life Science, USA). The reaction mixture was incubated at 41 °C for 1 h, followed by 5 min at 95 °C to inactivate the reverse transcriptase. The resulting cDNA was used for PCR with the sequence-specific primer pairs for IL-10. Amplification of the β-actin cDNA sequence was performed in a separate tube as the internal standard. The primers used were: (a) IL-10; sense, 5′-CTGCTCTTACTGACTGGCATGAG-3′, antisense, 5′-GACTCAATACACACTGCAGGTGT-3′: (b) β-actin; sense, 5′-TTCTACAATGAGCTGCGTGTGGC-3′, antisense, 5′-CTCATAGCTCTTCTCCAGGGAGGA-3′.

PCR amplification was performed in a 50 µl reaction mixture which contained 5 U of Taq polymerase, 50 µM dNTPs, PCR buffer, which contained 3 mM $MgCl_2$ (Takara Biochemicals, Japan) and 0.4 µM of each primer. After initial denaturation at 95 °C for 3 min, 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 for β-actin and 40 cycles for IL-10. The amplified PCR products were identified using electrophoresis of 10 µl aliquots on a 2% agarose gel and were stained with 0.5 µg/ml ethidium bromide. The products were visualized under UV light and photographed. The specificity of the amplified target sequences was confirmed by the restriction enzyme digestion that produced the predicted size (data not shown).

Quantitative digital image analysis of the PCR product

The photographs were scanned into a computer using an image scanner. The public domain NIH image software (Wayne Rasband, National Institutes of Health, USA and available through the Internet) was used for the quantitative digital image analysis of the band of the PCR product. The ethidium bromide staining in the digitized image produced a luminescence curve (densitogram). After the base line was added to remove the background, the area under the boundary line (curve and base line) was determined. The ratio of IL-10 to β-actin band intensity was calculated to normalize the determined value for IL-10 relative to that for β-actin to compare the samples of the skin specimens at 0–180 min after incision. The IL-10 to β-actin ratios were plotted against time after incision.

Cycle titration

An experiment on cycle number titration was performed using skin specimens from 60 min after incision. The RT-PCR procedure was performed under the same conditions as described. The PCR product was analysed after every 2 cycles, from cycles 22–32 for β-actin and cycles 32–42 for IL-10. The amplified products were separated on a 2% agarose gel and the band intensities were measured using the NIH image program. The logarithm of the determinations of the bands was plotted against the cycles.

Determination of wound vitality by RT-PCR for putrefied skin specimens

Detection of β-actin mRNA from putrefied skin specimens

To investigate the viability of mRNA change after postmortem, RT-PCR was performed using skin specimens at various phases of putrefaction. For this, 14 mice without incision were sacrificed and placed in an air conditioned room (temperature 24 °C, humidity 60%) and skin specimens were excised on days 0 to 13. The same RT-PCR procedure was used with the sequence-specific primer pairs for β-actin. The amplified products were visualized on agarose gel under UV light.

Determination of wound vitality

An incision was made on the dorsal skin of 48 mice with the same protocol and 12 mice were sacrificed at 0, 30, 60 and 180 min after incision. A 2-cm postmortem incision was performed on 12 separate mice after sacrifice and 12 mice without incision were used as controls. The 72 mice were put in the same air conditioned room as used previously. At 1, 2, 3 and 5 days postmortem, the skin specimens were excised from 3 mice with post-infliction intervals, from 3 postmortem-incised mice and from 3 mice without incision. The initial amount of IL-10 mRNA of these skin specimens was determined using the same procedure and the IL-10 to β-actin ratios were plotted against the postmortem interval.

Statistical analysis

The mean and standard deviations were calculated for the survival times after incision and for the postmortem intervals. The difference in the IL-10 to β-actin ratio of mice without incision and wounded mice was determined at the postmortem intervals. The significance was determined by T-test with $P < 0.05$ considered significant.

Results

Expression of IL-10 mRNA in situ

IL-10 mRNA was detected from 0 to 180 min after incision and the PCR bands for β-actin (internal standard) were not significantly different after incision (Fig. 1), which indicated that the efficiency of RNA extraction and RT-PCR was similar for the various skin specimens. Fig-

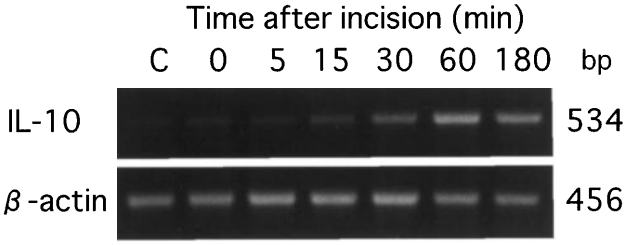

Fig. 1 RT-PCR analysis of IL-10 and β-actin mRNA expression at the wound site during mouse skin wound healing. The size in base pairs of the amplified cDNA fragments is indicated. Lane C indicates the results of mice without incision (control)

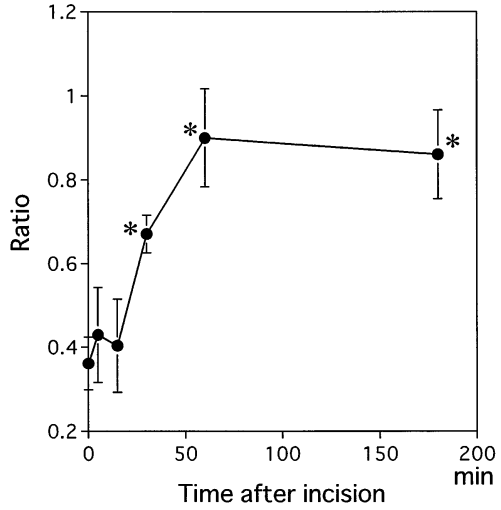

Fig. 2 Time-dependent expression of IL-10 mRNA during the early phase of skin wound healing. The results represent the mean ± SE of at least 5 mice per group. *$P < 0.05$ (vs. control)

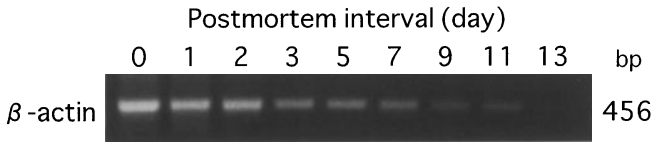

Fig. 3 Detection of β-actin mRNA from skin specimens with postmortem putrefaction by RT-PCR

ure 2 shows the relative change in mRNA expression for IL-10 from 0 to 180 min after incision. After 15 min there was a rapid increase in the amount IL-10 mRNA which peaked at 60 min. In comparison to the mean IL-10 to β-actin ratio of mice without incision (0.31 ± 0.11, mean ± SD), a significant increase ($P < 0.05$) in IL-10 mRNA occurred from 30 to 180 min.

Experiments on cycle titration revealed that the amplification reaction did not plateau within the number of cycles used in this study (30 cycles for β-actin and 40 for IL-10), especially not for the peak amount of IL-10 mRNA at 60 min after incision (data not shown).

Determination of wound vitality by RT-PCR for putrefied skin specimens

Amplified PCR products of β-actin cDNA for the putrefied skin specimens were detected on agarose gels from 0 to 11 days postmortem (Fig. 3), but the amount of β-actin mRNA decreased during the postmortem interval. No additional PCR product caused by the postmortem changes on mRNA could be detected.

The time-dependent IL-10 mRNA expression (as shown in Fig. 1) was maintained for 5 days postmortem (Fig. 4). Figure 5 demonstrates the time course of the IL-10 to β-actin ratios postmortem and the comparison with IL-10 mRNA expression in mice without incision revealed that a significant difference occurred in mice 30, 60 and 180 min post-incision on days 1, 2 and 3. The mean IL-10 to β-actin ratio increased 2 days postmortem and 30, 60 and 180 min post-incision. No significant difference in IL-10 mRNA expression occurred between the mice without incision and the postmortem-incised mice.

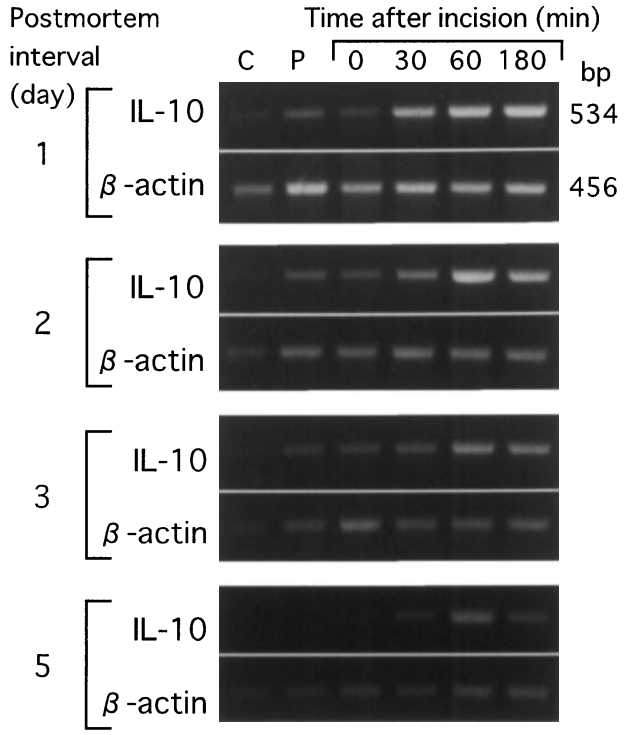

Fig. 4 RT-PCR analysis of IL-10 and β-actin mRNA expression from putrefied skin specimens. Lane C and lane P indicate the results of mice without incision and those of postmortem-incised mice, respectively

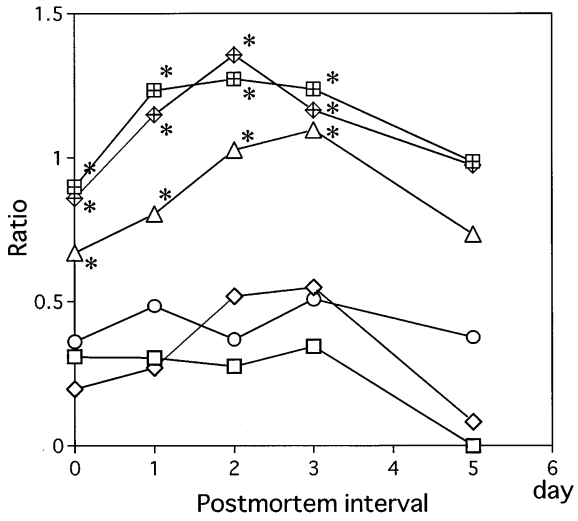

Fig. 5 Time course of IL-10 mRNA expression during the 5-day postmortem interval. The results represent the mean of at least 3 mice per group. □, control; ○, 0 min post-incision; △, 30 min post-incision; ⊞, 60 min post-incision; ⊕, 180 min post-incision; ◇, postmortem damage. *$P < 0.05$ (vs. control)

Discussion

The quantitative PCR method was used because of its sensitivity and simplicity. One method for the quantification of mRNA using PCR is to normalize the target gene expression relative to an internal standard gene such as β-actin that is expressed at a constant amount in the cell. To accurately determine the initial amount of mRNA, this method needs the PCR products of the target and internal standard gene to be quantified within the exponential range of each PCR amplification, as was performed for the cycle titration. Because the amount of band luminosity using the NIH image program reflects the initial amount of template DNA [4], an accurate determination of the IL-10 mRNA expression was performed using our experimental conditions.

Considering the early phase of skin wound healing in mice, the IL-10 protein level in situ peaked at 3 h after incision [3]. Our results demonstrated a correlation with the time course of IL-10 protein production, because the induction of IL-10 mRNA occurs before the production of IL-10 protein. The rapid increase of IL-10 mRNA suggests that IL-10 contributes to the acute inflammatory phase of skin wound healing by suppressing the inflammatory response.

To determine wound age and/or wound vitality, forensic pathologists have attempted to find useful biological markers that contribute to the healing process [1, 5–16]. These markers include several proteinase inhibitors, cytokines, extracellular matrix molecules etc. Recently, cell adhesion molecules and apoptotic cell death have also been investigated for the forensic application [17–19]. Most studies on wound age determination use the techniques of immunohistochemistry and molecular biology.

The basal cell synthesis of RNA has been used for wound examination. Buris [20] demonstrated that RNA synthesis at the wound site rapidly increased 15 min after wounding using ^{3}H-Uracyl-2 as the RNA precursor. Oehmichen and Lagodka [21] investigated time-dependent RNA synthesis during skin wound healing. Because of the lack of sensitivity of autoradiography, there has been no study on the use of very little, specific mRNA for forensic wound examination.

According to Oehmichen [22], if morphological and biochemical changes are to be used to analyse wounds as a marker of wound age or wound vitality, the following conditions should be demonstrated; (a) time dependency of the phenomena, (b) postmortem stability of the assay, and (c) postmortem time dependency. For wounds with a short post-infliction interval, in situ IL-10 mRNA expression fulfills these 3 conditions.

A low amount of IL-10 mRNA expression does not necessarily demonstrate that the damage was sustained postmortem, because IL-10 mRNA expression showed the maximum at 60 min after incision, followed by normalization at 24 h after the peak (unpublished observation). A postmortem increase of the IL-10 to β-actin ratio suggests that the mRNA induction mechanism functions in cells after somatic death [23], but no significant difference in IL-10 mRNA expression occurred between mice without incision and postmortem-incised mice. We consider the increased expression of IL-10 mRNA to indicate wound vitality in a skin specimen with putrefaction.

For the use of our results for forensic practice, we suggest that IL-10 mRNA expression is compared between the skin specimens of the wound and skin specimens proximal to the wound for the same cadaver. The latter should be a good internal control, because the skin specimens have a similar amount of putrefaction, and, therefore further study is necessary.

This study suggests the use of mRNA for forensic wound examination. Although there is putrefaction in the specimens, mRNA was determined by RT-PCR over a longer postmortem time course than expected. Because various proteins are synthesized after the induction of mRNA, mRNA might be a more sensitive indicator of wound vitality in forensic wound examination.

Acknowledgements The authors would like to thank Prof. Dr. med. M. Oehmichen (Lübeck) for his suggestions and critical reading of the manuscript and they are also very much obliged to Dr. T. Kondo and T. Minamino for their excellent assistance. This study was supported by Grants-in-Aid for General Scientific Research (T.O.; 08457146) from the Ministry of Education, Science, Sports and Culture of Japan, and presented in part at the 27th Annual Meeting of the Japanese Society for Wound Healing, Tokyo, December 1997. Dedicated to Prof. Dr. med. M. Staak on the occasion of his 65th anniversary.

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