JMS 2023; Vol 6(1):121-126
UDC:616-001.4-037
DOI: 10.55302/JMS2361121b

TIME-DEPENDENT mRNA IL-1β EXPRESSION-A PRELIMINARY STUDY FOR POSSIBLE WOUND AGE MARKER

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Abstract

Accurate estimation of wound age is a very important task in forensic medicine field, because it can helps in reconstruction of crime scene. IL-1β is proinflamatory cytokine and plays a major role in wound healing process. The aim of the study is to develop a method for the reliable estimation of dermal injury age. In this study the expression of IL-1β mRNA was evaluated by real-time PCR at skin human wounds with different post-injuried period.

We collected 27 human skin wound samples from forensic autopsy cases at our Institute. In our study were include samples from lacerations, incised wounds and stab wounds, with a known time of injury and death.

Wound samples were divided into five groups: control group (n=5); first group consisted of cases with immediate death (n=7); second group consisted of cases with survival from 1 hour to 6 hours (n=5); the third group comprised cases with survival from 6 hours to 72 hours (n=3); the fourth group comprised cases with survival from 72 hours to 168 hours (n=7). In this study we detected increased expression levels of IL-1 β mRNA in the second group with survival time from 1 to 6 hours.

Keywords: IL-1β; wound age; Real-time PCR; autopsy cases; human dermal injuries

Introduction

Accurate estimation of wound age is a very important task in forensic medicine, because it can helps in reconstruction of crime scene. Also, the determination of wound age is very important because the expert opinion of the forensic pathologist can contribute to legal authorities.

The forensic pathologist is expected to give answers regardingthe time of infliction, the survival time after injury and therelationship between the injury andthe incident [1-8].

Wound healing is a dynamic and complex process, which involves reactions and interactions between various cell types and mediators. The wound healing process is divided into four phases. The first phase, coagulation and hemostasis, begins immediately after wounding. Its objective is to prevent exsanguinations. It is a way to keep vascular system intact. Inflammation is the second phase of wound healing and it begins shortly after wounding.

The goal of this stage is to establish immune barriers against microorganisms. The proliferative phase is characterized by angiogenesis, granulation tissue formation and reepithelization. The last stage, the phase of maturation, is liable for development of epithelium and final scar tissue formation [7-11].

Each of these phasesinvolve complicated but well-organized and time-dependent events that occur at the site of injury. All these changes lead to changes in the expression of genes involved in the wound healing process [6,12].

Cytokines are small secreted proteins released by a broad range of cells, including activated lymphocytes and macrophages, with a specific effect on the interactions and communications between cells [3,13,14].

Cytokines are closely involved in a variety of biological process of the immune, central nervous, endocrine and hematopoietic systems.IL-1, IL-6 and TNF-α are proinflammatory cytokines, which are constitutively expressed in uninjured skin and passively released after injury [15].

IL-1β is a proinflammatory cytokinethat plays a major role in the wound healing process. Il-1 primarily originate from macrophages, keratinocytes, monocytes and neutrophils and is released as a

response tocellular damage caused by injury. The primary function of IL-1β is to promote and mediate inflammation, by stimulating the production of multiple other cytokines and chemokines involved in the wound healing process [15,16].

The aim of this study was to develop a method for reliable estimation of dermal injury age. The expression of IL-1β mRNA was evaluated by real-time PCR in skin human wounds with different post-injury period. We also assessed IL-1β as a possible marker for the wound age determination.

Material and method

Samples and collection

We collected 27 human skin wound samples from forensic autopsy cases at our Institute. Samples from lacerations, incised wounds and stab wounds, with a known time of injury and death were included in our study. The postmortem interval was within 24 h from wounding.

Subjects aged from 20 to 60 years were included and those with metabolic disorders, dermal diseases and severe malnutrition were excluded. Fullthickness tissue specimens of wound sites (1-2 cm in length and 0.2-0.3 cm in width) obtained from the bodies were examined as well as intact skin samples of equal size that served as a control group.

Samples were immediately frozen at-96.6^{o}C with R134a tetrafluoroethane (CF3CH2F). Frozen specimens were stored in plastic cryotubes of 2 ml volume in a freezer at -80^{o}C until the day of molecular biological analysis.

Extraction of total RNAs and real-time PCR

The first step in our study was homogenization of the skin samples. We used a mechanical homogenization procedure with pestlesand tubes. The frozen skin samples were placed in a sterile 1.5 ml tube (Biomasher) and 400 μL of RB Buffer from MagCoretriXact kit and 4μL β-mercaptoethanol were added. During the homogenization process, the tubes were submersed in an ice bath.

The total RNA was automatically extracted from skin injury samples (approximately 100 mg) with MagCore Automated NucleicAcid Extractor Plus II (RBC Bioscience, Taiwan) using TriXact kit. The isolated RNA was DNase treated using PureLink DNase, to remove any traces of genomic DNA.The concentration of freshly isolated total RNA was quantified using Qubit® 2.0 Fluorometer.

A reverse transcription of the total RNA was performed using aHigh-Capacity cDNA Reverse Transcription Kit. A cDNA synthesis was performed in a 2 μl 10x RT Buffer, 0.8 μl 25x dNTP Mix (100 mM), 2 μl 10x RT Random Primers, 1 μl RNase Inhibitor, 3.2 μl RNase free H2O , 1 μl MultuScribe Reverse Transcriptase and 10 μl isolated RNA. The reaction mixture was incubated at 25 C for 10 minutes, followed by 37 C for 120 min. and in the end 85 C for 5 minutes.

The synthesized cDNA was then amplified in a PCR reaction to detect IL-1 and GAPDH, which was used as the endogenous gene. The real-time PCR procedure was repeated three times for each sample.

The obtained complementary DNA was analyzed fordetermining the cycle threshold (C_T) level of transcripts of interest and C_T level of housekeeping gene in order to assess the relative abundance of the transcripts in each of the phases of injured tissue. Real-time PCR (polymerase chain reaction in real time) was accomplished by application of commercial reaction mixture HOT FIREPol®EvaGreen® qPCR Mix Plus at 7500 Real-Time PCR System.

At this point, one Real-time PCR reaction was made to each sample for examined transcript (target) and one Real-time PCR reaction for the reference gene (housekeeping). The expression of 1 target genes, IL-1 β, was analyzed in this study. GAPDH gene was analyzed as a housekeeping gene. Primers for the examined genes were designed according to literature data (Table 1). For each of the special targets, specific primers for amplification were used.

Statistical analysis of results

The results obtained from RT-PCR analysis, that is, the C_T levels of transcripts of interest and C_T levels of housekeeping genes were calculated with the formula 2^(-ΔΔCt). The values obtained were statistically analyzed with the Pearson correlation coefficient and one way ANOVA.

Table 1. qRT-PCR Primer Sequences

Gene	Primer		References
GAPDH	*Forward* *Reverse*	TGCCAAATATGATGACATCAAGAA GGAGTGGGTGTCGCTGTTG	17
IL-1β	*Forward* *Reverse*	AGCTACGAATCTCCGACCAC CGTTATCCCATGTGTCGAAGAA	18

GAPDH, glyceraldehydes-3-phosphate dehydrogenase; IL-1β, interleukin 1 beta

Results

The study included 27 wound samples, lacerations, incisional and stabwounds. Wound samples were divided into five groups: control group (n=5); thefirst group consisted of cases with immediate death (n=7); the second group consisted of cases with survival from 1 hour to 6 hours (n=5); the third group comprised cases with survival from 6 hours to 72 hours (n=3); the fourth group comprised cases with survival from 72 hours to 168 hours (n=7).

There was a significant difference in the expression levels of IL-1β mRNA between the control group and wound groups.

The study detected increased expression levels of IL-1 β mRNA in the second group with survival time from 1 to 6 hours (Table 2, Figure 1). In comparison with the control group, the levels of IL-1 β were lower in the first group (group which consisted of cases with immediate death) and in the third group (6-72 hours survival time). In the fourth group, with survival time between 72-168 hours,increased expression of IL-1 β levels was detected compared to the control group.

Table 2. Relative quantity of IL-1β in wounds

Group	Survival time	IL-1 β
Control	/	0.1
First group	0-1 h	0.1 ± 0.17
Second group	1-6 h	0.3 ± 0.69
Third group	6-72 h	0.1 ± 0.20
Fourth group	72-168 h	0.2 ± 0.17

Note: Data are presented as means ± SD, $p < 0.05$

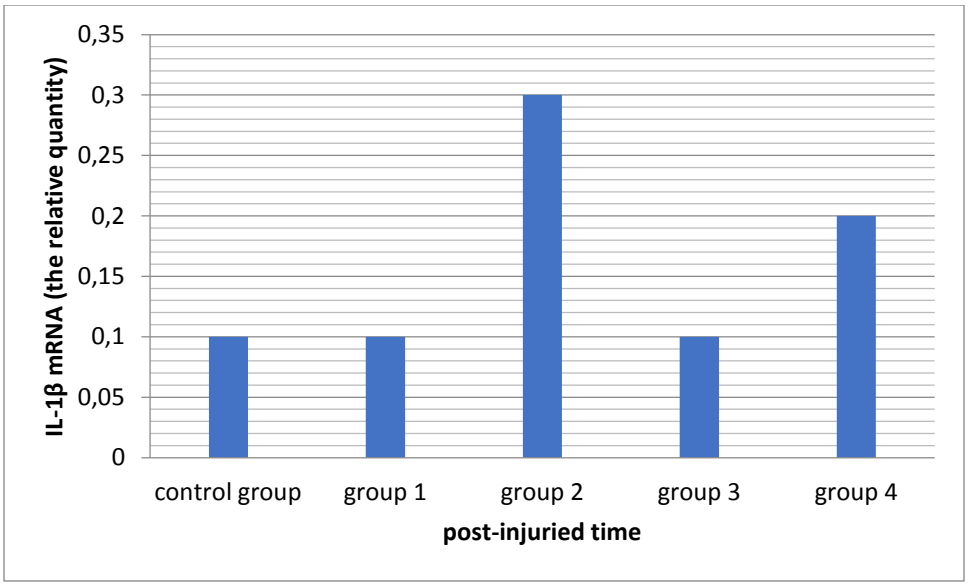

Figure 1. Real-time PCR analyses of IL-1β mRNA expression in wounds

Control group – intact skin (n=5), Group 1 - cases with immediate death (n=7), Group 2 - cases with survival from 1 hour to 6 hours (n=5), Group 3 - cases with survival from 6 hours to 72 hours (n=3), Group 4 - cases with survival from 72 hours to 168 hours (n=7).

Discussion

The determination of skin wound age continues to pose a challengeto the medico-legal practice [19]. To date, many techniques have beenused to determinethe age of injuries in skin specimens obtained during autopsy including histological and immunohistochemical methods.

There are studies conducted on live individuals, volunteers, whichactually allow for a more precise determination of the wound age, but all have beenfaced with ethical issues [20].

Apart from the morphological methods, research has been conducted with the application of biochemical methods for the evaluation of vital markers. By application of immunological tests, such as ELISA, significant results have been achieved in the analysis of proinflammatory cytokines [8,15,21].

The molecular analyses allow for a more detailed understanding of cell activation and mediator synthesis in different phases of the wound-healing process [22].

IL-1β is a cytokine that is a member of the Interleukin 1 family. Cytokines are primarily synthesized in the activated macrophages as a reaction to cell damage due to skin injury. The main function of IL-1β is to encourage inflammation through the stimulation of the production of other mediators that partake in the process of wound healing. IL-1β has additional functions, such as a role in cell proliferation, differentiation, adhesion, and apoptosis.

The studies conducted for identification of IL-1β as a potential marker for wound agedetermination have shown that there is an expression of IL-1β starting from the intermediary period of the inflammatory phase to the initial stages of the proliferation phase [23].

In studies conducted on experimental animals, a significant expression of IL-1β levels were detected in the time period of 9 hours to 5 days after wounding. Similar results were achieved in the studyconducted by Takamiya et al. (2009) on human skin wound specimens (live or autopsied individuals). In both of the previously mentioned studies, a second peak of IL-1β levels was noted on the 10th day after the initial injury [24].

In a studyconducted by Sato et al. on experimental animals, during which incise wound specimens had been collected, reverse transcription PCR was used to determine the expression of mRNA

for the proinflammatory cytokines IL-1a, IL-1β, IL-6 и TNF-α. It was discovered that IL-1β reached peak levels between 48 and 72 hours [25].

In a study by Birinciogluet al., 22 skin wound samples were obtained during autopsy, and the expression of mRNA of four cytokines and growth factors was analyzed. The results showed that IL-1β mRNA did not reach statistically significant levels in any of the examined wound groups compared to the other markers and the control group [3].

Palagummianalyzedthe expression of mRNA for a large number of cytokines and growth factors. The study was conducted on live individuals, volunteers, by swabbing incise wounds, and it was shown that the expression of IL-1β was significantly elevated in the specimens that were 12 hours old [7].

In our study, the expression levels of IL-1β mRNA weredetected in skin wound specimens that were 1 to 6 hours old. The results obtained are similar to those from other studies, with reference to the function of IL-1β in the process of wound healing. In other studies, where elevated levels of IL-1β were noted on the 4th day after the wound infliction, the results cannot be considered accurate, as they were due to the different manner of collecting the specimens. IL-1β is synthesized in the activated macrophages as an inflammatory response of the mediators, hence, its expression can only be detected in the inflammatory phase.

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