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Temporal expression of wound healing-related genes in skin burn injury

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ABSTRACT

Determination of the age of burns, as well as of wounds induced mechanically, is essential in forensic practice, particularly in cases of suspected child abuse. Here, we investigated temporal changes in the expression of 13 genes during wound healing after a burn. The expression of cytokines (IL-1β, IL-6, IL-10, TNF-α, and IFN-γ), chemokines (KC, MCP-1), proliferative factors (TGF-β, VEGF), proteases (MMP-2, 9, 13) and type I collagen in murine skin was examined by real-time PCR at 3, 6, 9, and 12 h and 1, 2, 3, 5, 7, and 14 days after a burn. Based on macroscopic and histological appearance, the healing process of a burn consists of 3 phases: inflammatory (from 3 h to 1 day after the burn), proliferative (from 1 to 7 days), and maturation (from 7 to 14 days). Expression of IL-1β, IL-6, TNF-α, IFN-γ and KC increased significantly in a biphasic pattern from 3 or 6 h to 12 h or 1 day and from 3 or 5 days to 7 days. Expression of MCP-1 increased significantly from 6 h to 5 days. Expression of both IL-10 and TGF-β increased significantly from 12 h to 7 days. Expression of VEGF, MMP-2, MMP-13 and type I collagen increased significantly from 3 days to 7 or 14 days. Expression of MMP-9 increased significantly from 6 h to 14 days. Our results suggest that evaluating the expression of a combination of these genes would enable the exact estimation of the age of a burn.

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1. Introduction

Child abuse and neglect are recognized as serious medical and social problems in advanced and developing countries. In cases of death as a result of child abuse, autopsies typically reveal plural skin injuries over the whole body with varying wound ages and types [1]. Forensic pathologists need to be able to evaluate the causal relationship between death and wounds, and estimate the age of each wound in order to determine the time period over which the children were maltreated. Burn injuries, mostly caused by a cigarette or boiling water, are also commonly observed in child abuse cases [1]; therefore, age determination of burn injuries as well as of mechanically-induced wounds is necessary in these cases.

The healing of a skin wound is an orchestrated process consisting of 3 sequential phases: inflammatory, proliferative, and maturation. These phases proceed via complicated interactions between various cell types [2]. A number of studies have demonstrated that a range of cytokines, chemokines, growth factors, and proteases are closely involved in the healing process [2,3]. In the forensic field, there have been many studies on the expression of these molecules in the skin for determination of the age of mechanically-induced wounds (by sharp or blunt objects) using animal experiments and autopsy samples [4–11]. However, there is much less information on determining the age of skin burns, and most of this is derived from classic macroscopic observations (e.g. scar formation). In this study, we examined temporal changes in the expression of 13 genes related to wound healing in order to obtain a means of estimating the age of burn wounds.

2. Materials and methods

2.1. Antibodies

The following monoclonal or polyclonal antibodies (mAb or pAbs) were used: rabbit anti-MPO pAbs (Thermo Fisher Scientific, Fremont, USA); rat anti-mouse F4/80 mAb (Dainippon Pharmaceutical Company, Osaka, Japan); anti-mouse matrix metalloproteinase (MMP)-9 mAb (Abcam, Cambridge, United Kingdom); biotinylated goat anti-rabbit IgG pAbs, biotinylated goat anti-rat IgG pAbs (Santa Cruz Biotechnology, Santa Cruz, CA); cyanine dye 3 (Cy3)-conjugated donkey anti-mouse IgG pAbs; and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rat or rabbit IgG pAbs (Jackson ImmunoResearch Laboratories, West Grove, PA).

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2.2. Mice

Pathogen-free 8–9-week-old male BALB/c mice were obtained from SLC (Shizuoka, Japan). All mice were bred and housed in a temperature-controlled (23 ± 2 °C) environment with a 12 h light/12 h dark cycle. They were fed with standard feed and given water ad libitum. All animal experiments were approved by the Ethics Committee for Animal Experimentation at Kagoshima University.

2.3. Burn injury procedure

Full-thickness burns were induced as described previously [12,13]. Briefly, under inhalation anesthesia with isoflurane, the dorsal skin of mice was exposed to a heated (100 °C) circular metal plate (2.0 cm in diameter) for 5 s. The mice were resuscitated immediately with an intraperitoneal injection of 0.05 ml/g body weight of saline. At 3, 6, 9, and 12 h and 1, 2, 3, 5, 7, and 14 days ($n = 4$ in each group) after the burn, mice were sacrificed by deep anesthesia, and skin samples were collected. Control skin was collected from non-burned mice ($n = 4$). The skin samples were immersed in RNA Later (Perkin-Elmer Applied Biosystems, Foster City, USA) and stored at −80 °C for the extraction of RNA; a part of each sample was fixed in 4% formaldehyde buffered with phosphate-buffered saline (PBS; pH 7.2) for histopathological analyses.

2.4. Histopathological analyses

Formaldehyde-fixed skin samples were embedded in paraffin. Six micrometer sections were cut and stained with hematoxylin and eosin (HE) or Masson's trichrome for the detection of collagen deposition. Immunohistochemical analyses were performed for the evaluation of leukocyte subsets as described previously [14]. Briefly, deparaffinized sections were immersed in 0.3% H_2O_2-PBS for 30 min to block endogenous peroxidase activity. The sections were incubated with rabbit anti-MPO pAbs (ready-to-use) as a marker of neutrophil or rat anti-F4/80 mAb (1:50) as a marker of macrophage at 4 °C for 14 h. Thereafter, the sections were incubated with biotinylated goat anti-rabbit IgG (1:100) or anti-rat IgG (1:100) at 18 °C for 1 h. After rinsing in PBS, the sections were further incubated with LSAB2 (Labeled StreptAvidin–Biotin; Dako Cytomation) at 18 °C for 30 min, and positive signals were visualized using 0.02% 3, 3′-diaminobenzidine, followed by nuclear staining with hematoxylin.

2.5. Double-color immunofluorescence analysis

Double-color immunofluorescence analysis was conducted as described previously [14] to identify the types of cells expressing MMP-9. Briefly, deparaffinized sections were incubated in a combination of anti-MMP-9 mAb (1:100) and anti-MPO or anti-F4/80 Abs at 4 °C for 14 h. After incubation with fluorochrome-conjugated secondary Abs (15 mg/ml) at 18 °C for 1 h, the sections were observed under fluorescence microscopy.

2.6. Extraction of total RNA, reverse transcription and quantitative real-time PCR

To examine the expression of 13 genes related to wound healing in skin samples, quantitative real-time PCR analyses were performed as descried previously [15,16]. Total RNA was extracted from the skin samples using ISOGEN (Nippon Gene, Toyama, Japan) as described previously [17]. One microgram of total RNA was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara Bio Inc., Otsu, Japan). Thereafter, the generated cDNA was subjected to real-time PCR analysis using SYBR Premix Ex Taq (Takara Bio Inc.) with specific primer sets (Supplementary Table 1). The expression levels of target transcripts are given as the ratio of the target normalized against the endogenous reference (18Rps).

2.7. Statistical analysis

The means and standard deviations (SDs) were calculated for all data. Statistical significance was evaluated using one-factor analysis of variance (ANOVA), followed by Turkey–Kramer's test. $P < 0.05$ was accepted as significant.

3. Results

3.1. General findings from burned skin

Representative macroscopic and histopathological images of burned skin at different time intervals are shown in Fig. 1. From 3 h to 1 day after the burn, edematous changes of skin (corresponding to formation of a fluid-filled blister in a human burn) were observed macroscopically at the margin of the burned area (Fig. 1a). Histopathological analyses revealed interstitial retention of fluid at the dermis (Fig. 1d) and massive infiltration of neutrophils (Fig. 1j). From 1 day to 7 days after the burn, macroscopic examination indicated gradual improvement to the edematous changes (Fig. 1b). Histopathologically, a reduction of interstitial fluid (Fig. 1e) and apparent cellular infiltration, including neutrophils (Fig. 1k), macrophages (Fig. 1n), and fibroblasts (Fig. 1e) were observed. From 7–14 days after the burn, re-epithelization was observed from the marginal region of the burn (Fig. 1c). Histopathological analyses revealed progressive angiogenesis, proliferation of fibroblasts (Fig. 1f) and deposition of extracellular matrix (ECM) including collagen (Fig. 1i). The changes to the skin from 3 h to 1 day correspond to the inflammatory phase, those from 1 to 7 days to the proliferative phase, and those from 7 to 14 days to the maturation phase of the healing process of a skin wound.

3.2. Expression of genes related to wound healing

The expression of the genes examined in this study was altered after the burn and displayed a range of temporal patterns (Fig. 2). IL-1β, IL-6, TNF-α, IFN-γ and KC showed a significant increase in expression with a biphasic pattern from 3 or 6 h to 12 h or 1 day and from 3 or 5 to 7 days (Fig. 2a–e). Expression of MCP-1 increased significantly from 6 h to 5 days (Fig. 2f), while IL-10 and TGF-β showed a significant increase from 12 h to 7 days (Fig. 2g, 2 h). VEGF, MMP-2, MMP-13 and type I collagen showed a significant increase in expression from 3 to 7 or 14 days (Fig. 2i–l). Expression of MMP-9 increased significantly from 6 h to 14 days (Fig. 2m). These results are summarized in Fig. 3.

4. Discussion

Our study shows that a burn injury caused an increase in the expression of several genes that encode proteins involved in tissue repair during the wound healing processes [11,16].

We identified a biphasic increase in expression of IL-1β, IL-6, TNF-α, IFN-γ (proinflammatory cytokines) and KC (chemokine) during the inflammatory (3 h to 1 day) and late proliferative phases (3–7 days). Since the encoded proteins play roles in the induction of inflammation and neutrophil chemotaxis during the inflammatory phase [20–26], and in angiogenesis, immunosuppression and production of chemotactic factors for macrophages at the late proliferative phase [26–28] in the healing of a skin wound, the levels of gene expression might also be expected to increase at both phases in a skin burn.

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