Mohammed A. Alzoghaibi*, Abdullah I. Al-Oraini, Ali I. Al-Sagheir and Ahmad M. Zubaidi

Temporal expression of IL-1β and IL-10 in rat skin, muscle, small bowel, and colon wounds: a correlative study

Abstract

Background: Cytokines play a major role in coordinated wound healing events. We hypothesized that rapid intestinal healing is due to an early upregulation of the pro-inflammatory cytokine interleukin-1β (IL-1β), followed by increases in the expression of the anti-inflammatory cytokine IL-10.

Methods: We characterized the time course of IL-1β and IL-10 release at four wounds (skin, muscle, small bowel, and colonic anastomosis) after surgery on 38 juvenile male Sprague-Dawley rats. The tissue samples of each site were harvested at 0 (control), 1, 3, 5, 7, and 14 days post-operatively (n=6–8 per group) and analyzed by enzyme-linked immunosorbent assay kits for IL-1β and IL-10.

Results: IL-1β expression peaked at days 5 and 7 in small bowel and colonic wounds when compared to skin or muscle. Similarly, IL-10 showed high expression in these time points in small bowel and colonic wounds. However, IL-10 showed the same expression in all time points in muscle and skin tissues except at day 1.

Conclusions: The high expression in IL-1β and IL-10 levels in small bowel and colon might explain the accelerated healing process in these wounds in comparison to skin and muscle tissues. Additional studies are required to determine whether IL-1β and IL-10 expression is the major factor defining site-specific differences in healing rates in different tissues. Understanding cytokine action in the wound healing process could lead to novel and effective therapeutic strategies.

Keywords: IL-1β; IL-10; rat intestinal wounds.

***Corresponding author: Mohammed A. Alzoghaibi,** PhD, Physiology Department and Center of Excellence in Biotechnology Research, College of Medicine, King Saud University, P.O. Box 2925, Riyadh 11641, Saudi Arabia, Phone: +966-506338400, E-mail: zzoghaibi@gmail.com

Abdullah I. Al-Oraini, Ali I. Al-Sagheir and Ahmad M. Zubaidi: Surgery Department, College of Medicine, King Saud University, Riyadh, Saudi Arabia

Introduction

Wound healing is a complex procedure that requires a highly regulated series of events including interactions between many cell types, production of different soluble factors, and growing of matrix components. Normal wound healing has four phases: hemostasis, inflammation, tissue formation, and tissue remodeling [1, 2]. The prime goal of wound repair is to achieve hemostasis, which represents the first step in wound healing.

The inflammatory phase begins after 6 h of tissue injury. This phase has important events, such as migration of activated neutrophils, macrophages, and lymphocytes from the circulation into the injured tissue. Neutrophils are the first leukocytes to infiltrate the injured tissue followed by monocytes [3]. Neutrophils are dominant in the wound site during the first days. In contrast, macrophages (activated monocytes) reached their maximum level at day 2 [4]. Chemokines selectively mediate the recruitment of neutrophils and monocytes in the affected area [5].

Sato et al. have shown that endogenous interleukin-10 (IL-10), an anti-inflammatory cytokine, inhibited the infiltration of neutrophils and macrophages into the injured tissue as well as expression of several chemokines and pro-inflammatory cytokines [6]. Another study has investigated the role of IL-10 in fetal scarless wound healing [7]. It showed that the absence of IL-10 in IL-10 knockout mice resulted in scar formation in fetal wounds that would otherwise heal scarless. These results suggested an important role of IL-10 in regulating the expression of pro-inflammatory cytokines in fetal wounds, leading to reduced matrix deposition and scar-free healing. In addition to the inhibitory effect of IL-10 on the expression of IL-6 and IL-8, IL-10 has also been shown to inhibit the infiltration of neutrophils, monocytes, and macrophages in fetal dermal wound healing via the inhibition of other chemokines such as macrophage inflammatory protein-1α and monocyte chemoattractant protein-1 [8].

IL-1β is a pro-inflammatory cytokine produced in a variety of cells including monocytes, activated macrophages, keratinocytes, and other epithelial cells [9]. IL-1β

might be thought to be deleterious in wound repair but it has been shown to be essential in the early phases of wound healing [10]. Anti-inflammatory cytokine such as IL-10 suppresses the production of pro-inflammatory cytokines such as IL-1β [11, 12]. Therefore, the balance between pro-inflammatory and anti-inflammatory cytokines is important in determining the wound healing process.

Based on the role of IL-10 in wound scarless healing process and the importance of lack of inflammatory phase and inflammatory mediators in accelerated tissue repair that is mediated by IL-10 [13], we chose to study the temporal expression of IL-10 in a well-validated model of wound healing, comparing IL-10 cytokine expression in skin, muscle, small bowel (small intestine), and colon wounds in rats. The second aim of this study is to answer the question of whether IL-1β expression in wound is negatively correlated to IL-10?

Materials and methods

Animals

Thirty-eight male Sprague-Dawley rats (200–250 g) were used. Each rat was housed individually in a single cage and placed in a room with controlled temperature and light/dark cycle (12 h/12 h). The animals were allowed ad libitum intake of normal rat pellet food. Thirty-eight animals underwent surgery, with 6–8 rats in each group; enzyme-linked immunosorbent assay (ELISA) was performed for IL-10 and IL-1β cytokines at the following time points: 0 (control), 1, 3, 5, 7, and 14 days. The studies were conducted according to a protocol approved by the Animal Care Committee of the College of Medicine, King Saud University.

Surgical procedure

The animals were anesthetized with 1%–2% inhalational halothane. A single preoperative prophylactic dose of cefazolin (30 mg/kg; Novopharm Limited, Toronto, ON, Canada) was administered subcutaneously. The abdomen was prepared, shaved, and entered through a midline incision. Transection with immediate reanastomosis was performed both at the terminal ileum (10 cm proximal to the ileocecal valve) and at the transverse colon (1 cm proximal to the splenic flexure). The techniques were similar to previously published methods [14, 15]. In brief, the anastomotic sites were chosen. Dissection was done carefully to maintain the arcade and the mesenteric marginal vessels at the site of the transection of the bowel. The vessels were carefully dissected away from the terminal ileum and the colon and the marginal vessels were ligated just at the transection site. A standardized end-to-end anastomosis was performed with 8–10 interrupted inverting sutures using 6-0 monofilament polypropylene (Prolene™, Ethicon, Parsippany, NJ, USA). Polypropylene was chosen because of its inert characteristics, minimizing the inflammatory reaction associated with resorption of an absorbable suture [14, 15]. The abdomen was washed out with sterile normal saline and closed in layers using interrupted sutures of 4-0 polypropylene in the fascia and skin.

After the procedure, rats were housed in single cages. The animals were allowed water immediately and solid food at 24 h after surgery (20 g/day). Daily oral intake and weight were recorded. At the time of sacrifice, rats were anesthetized with halothane/oxygen by nose cone. Midline cutaneous and fascial wounds were harvested for 5 mm on each side of the wound, dissecting the skin and fascia away from the underlying tissue followed by en bloc excision without disturbing the actual wound. A laparotomy was then performed. Both the small bowel and the colonic anastomosis were identified and gently dissected away from the surrounding tissues. Care was taken to avoid disrupting adhesions as much as possible. If the anastomosis was not intact, the animal was excluded from further study. A 5-mm segment from each side of each anastomosis was excised en bloc. The rats then were euthanized by intracardiac injection of 50 mg/kg sodium pentobarbital. All harvested tissues were wrapped in aluminum foil and immediately frozen in a liquid nitrogen bath. All frozen tissue samples were kept at –70°C until further processed for cytokine measurement.

Extraction of tissue proteins

Tissues were processed with modification according to the method reported by Takahashi and Das [16]. In brief, each tissue specimen was thawed, minced into 2-mm cubes, washed five times in five volumes of phosphate-buffered saline, pH 7.2, containing 2 mM phenylmethyl sulfanyl fluazide and 2 mM sodium azide. After centrifugation at 1000 *g* for 5 min, the tissue pellet was homogenized in the same buffer including 1 mM EDTA [5 vol (wt/vol)] on ice in a Polytron (Kinematic type PT 1020350D; Dispersing and Mixing Technology, Lucerne, Switzerland) at 5–10× speed for four 10-s intervals. Homogenates were centrifuged at 2000 *g* for 20 min to remove large tissue particles and recentrifuged at 13,200 *g* for 90 min to obtain a clear supernatant. The supernatants of each tissue specimen were further divided into 200-μL aliquots, labeled, and stored at –70°C. Before each experiment, the extracts were thawed and centrifuged at 13,200 *g* for 5 min to remove debris.

ELISA assays

The supernatants were quantitatively assayed for IL-10 and IL-1β (Assay Design, Ann Arbor, MI, USA), using a 96-well microtiter plate reader. The respective cytokine levels were determined in the different tissue samples by means of duplicated measurements requiring 50–200 μL of extract. Results were verified by using internal controls supplied with each kit, with a known concentration of the target protein. Optical density measurements were then verified against a standardized curve. The results were averaged at the end of the experiment and expressed in picograms per gram wet tissue weight.

Statistical analysis

The results of cytokines for all tissues were plotted on graphs against the time of sacrifice. Parametric one-way analysis of variance

(ANOVA) was used to compare each cytokine in the four different wound sites at the same time point. Repeated-measures ANOVA was used to compare the temporal expression of each cytokine in a specific wound over the period of the study. Post hoc testing was done using the Bonferroni-Dunn method, with $p<0.05$ considered as significant, using GraphPad Prism® Version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). The results were expressed as the mean±standard error of mean.

Results

All animals in this study were healthy, eating equivalent amounts of chow, and gaining weight over the study period. There was no visual evidence of dehiscence (abscess, fistula formation, or fecal contamination within the peritoneum) at the time of the harvest in any of the animals. Detection of IL-10 and IL-1β cytokine expression using ELISA technique was investigated at the selected time points (0, 1, 3, 5, 7, and 14 days). The results of the quantitative analyses differed considerably between the different wound samples and are reported separately for each wound tissue (skin, muscle, small bowel, and colon).

Initially, skin IL-10 levels showed similar patterns of expression at all selected time points except at day 3 that showed a significant reduction in IL-10 levels in comparison to day 5 (90.9±31.5 pg/g wt; 131.5±14.4 pg/g wt, respectively). At day 1 after wounding, skin IL-10 levels increased dramatically compared to other selected time points but were not significantly different (Figure 1A). Muscle IL-10 levels did not show any significant difference between the main time points (1, 3, 5, 7, and 14 days) (Figure 1B).

IL-10 showed a distinct expression pattern in the small bowel and colon wound sites in comparison to the other wound sites. For the small bowel wounds (Figure 1C), IL-10 levels were significantly higher at days 5, 7, and 14 in comparison to days 0, 1, and 3 (day 5: 127.8±27.1 pg/g wt; day 7: 128.3±33.8 pg/g wt; day 14: 123.8±42.1 pg/g wt; versus day 1: 48.7±34.4 pg/g wt; and day 3: 52±12.9 pg/g wt, $p<0.05$). IL-10 expression showed a similar pattern in colon wounds as in the small bowel (Figure 1D). IL-10 levels were significantly higher at days 5, 7, and 14 in comparison to days 0, 1, and 3 (133±17.7 pg/g wt, 114±41.3 pg/g wt, and 109±24.2 pg/g wt, respectively, versus day 1: 68.5±28.2 pg/g wt, and day 3: 72.2±10.3 pg/g wt, $p<0.05$).

Similarly, IL-1β levels showed a distinct expression pattern in the small bowel and colon wound sites in comparison to the other wound sites (Figure 2). On days 1 and 3, IL-1β levels were significantly lower than on day 5 and 7 ($p<0.05$) in both small bowel and colon wound sites. On day 14, IL-1β levels showed significant reduction than day 5 in colon wound site. Skin and muscle IL-1β levels did not show any significant difference between the main time points (1, 3, 5, 7, and 14 days) (Figure 2A and B).

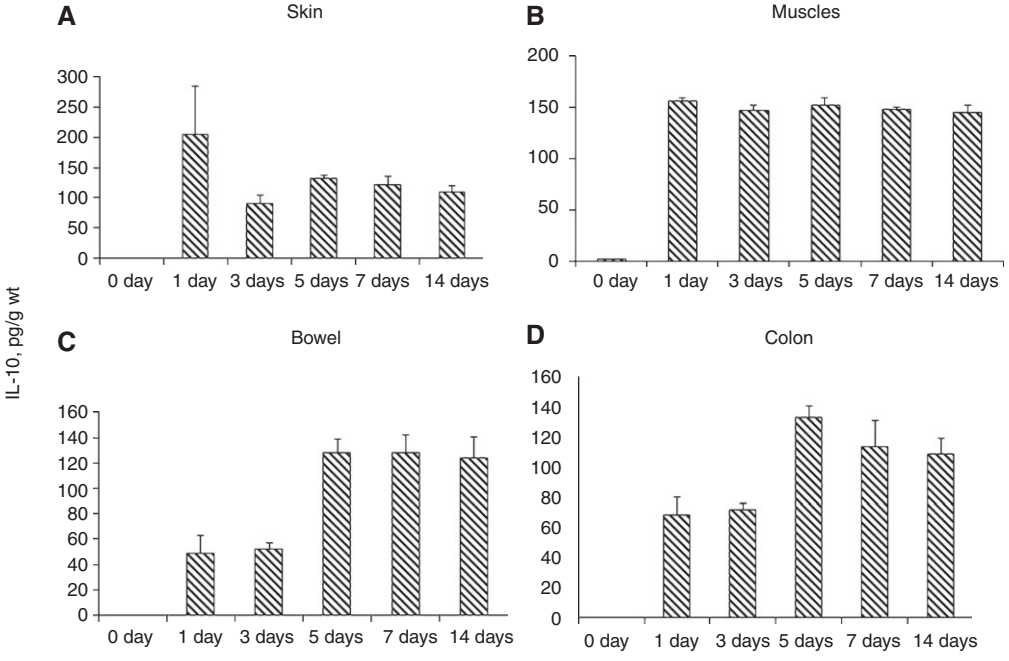

Figure 1 IL-10: time course of expression.
IL-10 profile in different wound sites over time. IL-10 levels were measured using ELISA and are expressed as pg IL-10/g wt of tissue. Data are expressed as mean±SEM, n=6–8 for each wound site at each time point.

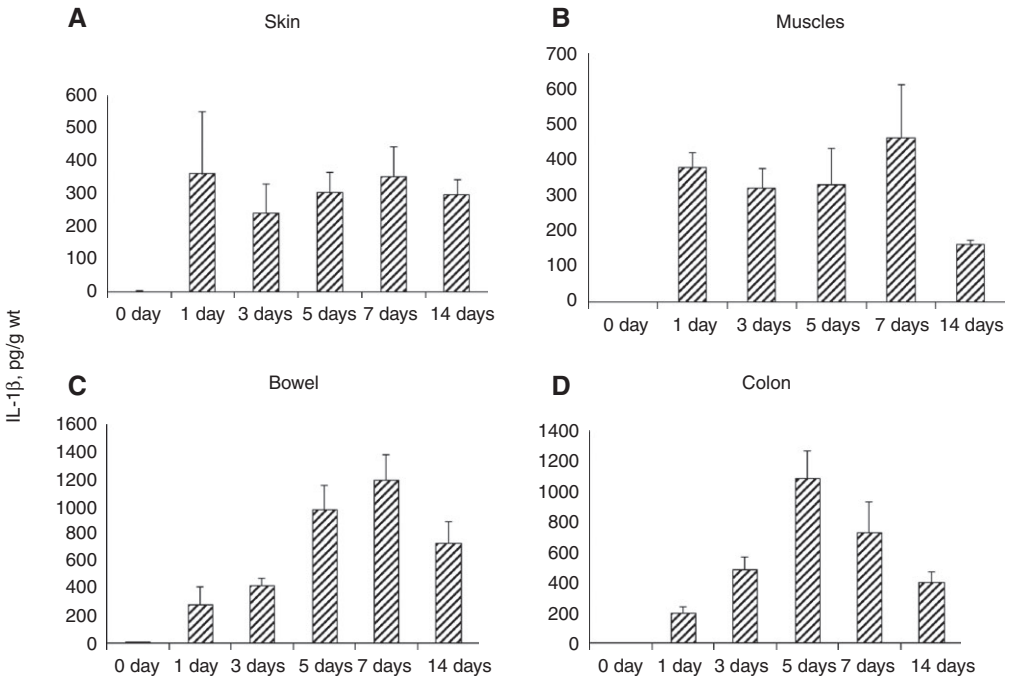

Figure 2 IL-1β: time course of expression.
IL-1β profile in different wound sites over time. IL-1β levels were measured using ELISA and are expressed as pg IL-10/g wt of tissue. Data: mean±SEM, n=6–8 for each wound site at each time point.

In order to know if there is any correlation between IL-10 and IL-1β levels in all tissue wounds, we used Spearman's correlation coefficient. We found a weak relation between IL-10 and IL-1β levels ($p<0.05$) (Figure 3).

Discussion

For optimal wound healing to occur anywhere in the body, an organized and complex cascade of cellular

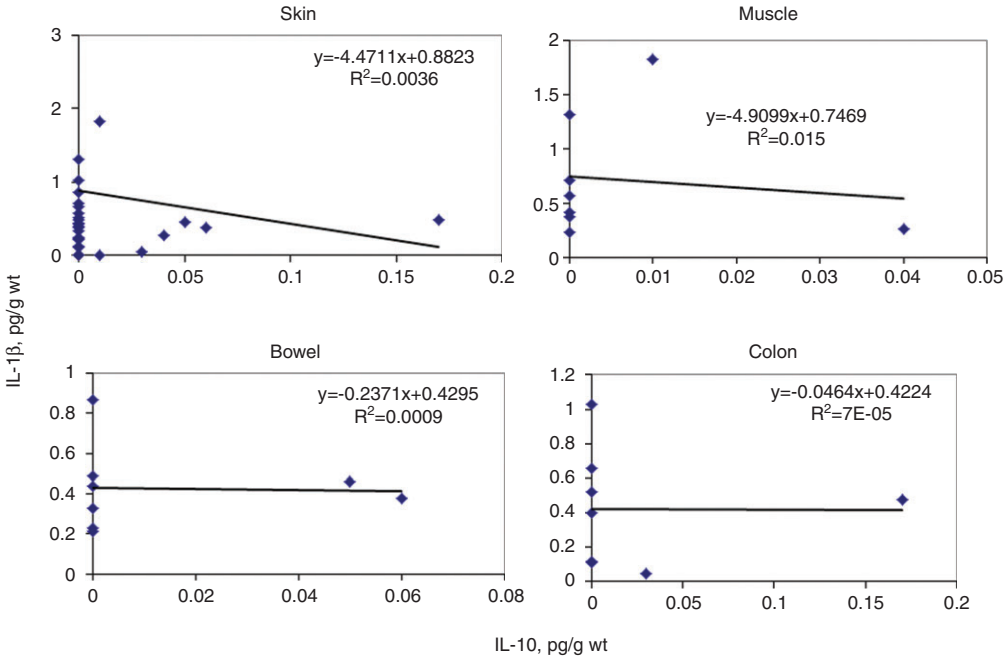

Figure 3 Correlation between IL-10 and IL-1β expression in different wound sites.
The figure shows no relation between IL-10 and IL-1β since $p>0.05$.

and biochemical events has to take place. Wound repair requires constant communication among the participating tissues. Inflammatory cells communicate with each other through cell-cell contact, cytokines, and growth factors [4]. In this milieu, cytokines and growth factors may have multiple functions that can initiate and influence the process of wound healing [5]. The spectrum of biological activity attributed to these factors includes mitogenic and chemoattractant properties, the ability to stimulate and release of other cytokines, the promotion of angiogenesis, and stimulation of extracellular matrix production [17].

Cytokines have been classified as pro-inflammatory and anti-inflammatory factors depending on the major functions that they serve [18]. Pro-inflammatory cytokines such as IL-1β, tumor necrosis factor-α, and interferon-γ might be thought to be deleterious in wound repair but they have been shown to be essential in the early phases of wound healing [19–21]. IL-1β is a pro-inflammatory cytokine produced in a variety of cells including monocytes, activated macrophages, keratinocytes, and other epithelial cells [22]. IL-1β is not usually constitutively expressed in healthy subjects [23]. It can activate neutrophils, upregulate adhesion molecule expression, promote the secretion of other pro-inflammatory cytokines, and further contribute to local inflammatory processes [24]. Attard et al. showed delayed closure of cutaneous wounds in IL-1 receptor deleted mice (IL-1R1−/−) [14].

IL-10 acts to suppress the activity of macrophages and reduce their cytokine production (IL-6 and IL-1β) as well as the T helper cytokine [25, 26]. A role for IL-10 in wound repair has been suggested by the results of several earlier studies.

Temporally, IL-10 expression follows the early pro-inflammatory cytokine response, likely to limit both the magnitude and the duration of the inflammatory response and allow the wound to evolve into a more proliferative healing phase [27]. IL-10 has been found to be an effective anti-scarring agent and reduced inflammation in experimental and clinical trials [7]. The findings of Henderson et al. have suggested that although not significantly altering collagen reorganization in mice, IL-10 has reduced the rate of wound contraction [28]. In contrast, Eming et al. have shown for the first time that IL-10 has important effects on tissue repair that delayed the closure of noncomplicated wounds in healthy animals. This effect was attributable to accelerated epithelialization as well as enhanced contraction of the wound tissue in the mutant animals [13].

Our data show that IL-10 has a significant expression on day 1 for the skin wound site and at all time points for the muscle wound site. However, IL-10 expression was significantly higher in days 5, 7, and 14 in both small bowel and colon wounds. Although healing of intestinal and skin wounds has similar sequence of events, there are differences in the details [29]. Furthermore, previous studies have shown that healing in intestinal wounds is proportionally faster than in skin [30, 31]. On the basis of the conclusion of Eming and colleagues that IL-10 can impede wound repair, our data might explain the faster healing of intestinal anastomotic wounds where we have less production of IL-10 until day 5 in comparison to skin and muscle incisional wounds. In this study, we aimed to correlate IL-10 and IL-1β levels in these four wound sites. Our data did not show any significant correlation between IL-10 and IL-1β in all wound tissues. It has been demonstrated that IL-10 inhibits IL-1β [11]. However, according to this study, IL-10 might participate in accelerating wound repair in bowel and colon wounds via other mechanisms.

In conclusion, the present study has shown an early expression of IL-10 in skin wound sites and late expression in bowel and colonic wounds, with no significant correlation with IL-1β. These findings might support the early healing process in intestinal anastomotic wounds via another pathway rather than the inhibition of pro-inflammatory cytokine IL-1β. Further studies should seek additional information about kinetics of changes in chemokine and cytokine levels postsurgery and the association of changes in chemokine and cytokine levels with the wound healing process. We hope that this present study provides a very good starting point for more detailed observations by other investigators to improve the understanding of the biology of wound healing as a guide for the development and use of cytokine/anti-cytokine and growth factor therapy in the preoperative period.

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Conflict of interest statement

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