Original Article

The Time Course of Cytokine Expressions Plays a Determining Role in Faster Healing of Intestinal and Colonic Anastomatic Wounds

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ABSTRACT

Objectives: Inflammation is critical in the early phases of wound healing. It has been reported previously that small intestinal and colonic wounds display a more rapid healing than those of other organs. However, the underlying mechanism has not yet been elucidated. Here we examined whether differences in the time course of specified cytokine expression, in colonic and small intestinal anastomotic lesions, might play a major role in this observation in comparison to lesions effecting skin and muscle tissue. **Materials and Methods:** Tissue lesions were applied to 36 male Sprague–Dawley rats. Tissue samples were harvested at 1, 3, 5, 7, and 14 days postoperatively with the levels of TNF-α, IL-6, and IFN-γ determined by ELISA-derived methods. **Results:** The characteristics of TNF-α, IL-6, and IFN-γ expression during the healing process for intestinal and colonic lesions were comparable. However, data differed significantly with that observed during healing of skin and muscle lesions. Intestinal and colonic lesions exhibited a significant and sustained increase in specified cytokine levels on day 5 to day 14 as compared with day 1 and 3. Skin and muscle lesions had random or unaltered cytokine levels throughout the study period. **Conclusion:** Differences in expression of cytokines TNF-α, IL-6, and IFN-γ indicate that these play an important role underlying the more rapid healing processes observed in small intestinal and colonic lesions.

Key Words: IFN-γ, IL-6, TNF-α, wound healing

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Insult to the integrity of skin, mucosal surface, or organ tissue results in the formation of a wound. Wounds can occur as part of a disease process or can have an accidental or intentional etiology.[1] With the occurrence of tissue injury, multiple cellular, and extracellular pathways are activated in a tightly regulated and coordinated fashion. The process of wound healing is separated into four distinct phases: Hemostasis, inflammation, proliferation, and tissue remodeling.[2] Inflammatory responses following tissue injury play an important role both in normal and pathological wound healing.[3] This observation has focused attention on the study of specific pro- and anti-inflammatory mediators, their interactions, and time courses, when involved in the processes of wound healing. The spectrum of biological activities attributed to these factors include mitogenic, chemotactic, angiogenic, and of the production of extracellular matrix components.[4] At each phase of wound healing, both local and recruited cells are activated, resulting in the production of these factors, which subsequently modulate the ongoing process of wound repair.[5]

In general, cytokines have been classified as proinflammatory and anti-inflammatory, depending on the major functions that they serve. Growth factors are considered healing promoters. However, the significance of each factor is not always obvious; pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), might be thought to be deleterious in wound repair but their expression has been observed in the early phases of wound healing implying an important role.[6-9]

TNF-α is released primarily by the macrophage monocyte lineage of cells and is essential in initiating the immune

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cascade during the host response to injury. Previous authors have shown that systemic TNF-α inhibition leads to qualitative impairments in cutaneous and intestinal wound healing with alterations in collagen deposition.[10,11] IFN-γ within the wound environment is primarily released by natural killer cells (NK cells), T lymphocytes, and macrophages.[10,12] Its predominant effects include resident macrophage and neutrophil activation in order to increase cytotoxicity, and an intensification of the local inflammatory response by increasing IL-1β, NO, and TNF-α production in macrophages.[13,14] IFN-γ furthermore plays an important role in the remodeling of wound tissues; where overproduction of this factor locally can decrease wound contraction and collagen synthesis.[13,15] Administration of IL-6 to Wister rats has been shown to significantly diminish the chronic inflammatory process in colon tissue and exhibited markedly less granulation within immature collagen formation.[16] In contrast, in colon anastomotic healing in rats, increased levels of IL-6 were detected, which led to improved healing and mechanical stability during the early postoperative period.[17] IL-6 levels were also found to be elevated in the lung, liver, and kidney wounds 24 h after anastomosis and a lack of IL-6 expression impaired wound healing.[18]

To determine the role and the significance of the time course of expression of inflammatory cytokines in anastomotic wound healing, we measured the IL-6, TNF-α, and IFN-γ levels in skin, muscle, small intestine, and colonic anastomotic wounds in Sprague-Dawley rats at different time points.

MATERIALS AND METHODS

Animals and surgical procedure

This study was conducted in accordance with the guidelines and protocols set by the Animal Care Committee of the College of Medicine, King Saud University (KSU). This study was performed at KSU, College of Medicine, between April 2009 and December 2012. Thirty-six male Sprague–Dawley rats weighing 200–250 g were used in the study. Rats were divided into six groups with each consisting of six animals. Rats were maintained in a room with controlled temperature and light/dark cycle (12 h/12 h) allowing intake of normal rat chow *ad libitum*. Animals were anesthetized with 1%–2% inhalational halothane. A single preoperative prophylactic dose of cefazolin (30 mg/kg; Novopharm Limited, Toronto, Ontario, Canada) was administered subcutaneously. The abdomen was prepared, shaved, and dissected 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 the same to previously published methods.[19,20] 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 adopted because of its inert characteristics thus minimizing any inflammatory reaction associated with resorption of unabsorbable suture material. 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 postsurgery (20 g/d). Daily oral intake and weight were recorded. At the time of sacrifice, rats were anesthetized with halothane/oxygen using a nose cone. Midline cutaneous and muscle wounds were harvested including a 5 mm margin on each side of the wound, with dissection of the skin and muscle away from the underlying tissue and followed by an en bloc excision without disturbing the actual wound. A laparotomy was then performed. Both the small intestine and the colonic anastomoses were identified and dissected away gently 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 were then killed by an intracardiac injection of 50 mg/kg sodium pentobarbital. All harvested tissues were wrapped in aluminum foil and frozen immediately in a liquid nitrogen bath. All frozen tissue samples were kept at −80°C until further processing for cytokine measurement.

Extraction of tissue proteins

Tissues were processed according to a previously reported method.[20,21] In brief, each tissue specimen was thawed, minced into 2 mm cubes, washed five times in 5 vol. of phosphate-buffered saline (PBS), pH 7.2, containing 2 mM phenylmethylsulfonyl fluoride 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 PT, Dispersing and Mixing Technology; Lucerne, Switzerland) at 5 to 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. Supernatants of each tissue specimen were further divided into 200 mL aliquots, labeled, and stored at −70°C. Prior to each experiment, the extracts were thawed and centrifuged at 13,200 g for 5 min to remove debris.

ELISAs

The supernatants were quantitatively assayed for IL-6, TNF-α (Assay Design, Ann Arbor, MI, USA) and IFN-γ (Biosource, Camarillo, CA, USA), with concentrations following the manufacturer's instructions. Optical density was measured

at 450 nm. The respective cytokines levels were determined in different tissue samples by means of duplicated measurements requiring 50–200 μL of extract. Results were standardized by using internal controls supplied with each kit, with a known concentration of the target protein.

Statistical analysis

Results of cytokine and growth factor measurements for all tissues were plotted on graphs against the time of sacrifice. One-way analysis of variance (ANOVA) was used to compare each cytokines or growth factors in the four different wound sites at the same time point. ANOVA repeated measures were used to compare the temporal expression of each factor in a specific wound over the study period. *Post hoc* testing was performed using the Bonferroni and Dunnett T3 for multiple comparison, with $P < 0.05$ being considered significant; SPSS version 19 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp.) was used to perform the analysis. Results were expressed as mean ± standard error of mean.

RESULTS

Following the initial surgery, all animals were found to be healthy, and had no significant differences in their eating behavior and the amounts of chow consumed by the operated rats, compared with sham control. Weight gain of all the rats was also found to be identical throughout the study period and was comparable to that of the control. No visual evidence of dehiscence (abscess, fistula formation, or fecal contamination within the peritoneum) was reported at the time of the harvest in any of the animals.

IL-6 levels

The IL-6 levels in the skin, muscle, colon, and small intestine measured at indicated time points are presented in Figure 1. The IL-6 levels in the skin tissues measured on day 1, 3, 5, 7, and 14 were 211.3, 175.3, 232.7, 216.4, and 180.9 pg/mL, respectively. No significant change in the IL-6 levels was found among the studied durations. In muscle tissue, the IL-6 levels 1 and 3 days postsurgery were 361.6 and 282.3 pg/mL, respectively, which significantly fell to 247.9 ($P < 0.05$), 259 ($P < 0.05$), and 206.9 pg/mL ($P < 0.01$) after 5, 7, and 14 days, respectively. This decrease is significantly different compared with levels measured on day 1. In colon tissue, the IL-6 levels on day 1 and day 3 postwounding were 118.4 and 144.1 pg/mL, respectively. These levels significantly peaked to 236.6 ($P < 0.01$), 199.8 ($P < 0.05$), and 186.8 pg/mL ($P < 0.05$) at 5, 7 and 14 days respectively, after the surgery. In small intestine, the IL-6 levels followed the trend as found in the colon where the levels on day 1 and day 3 were 97.8 and 105.3 pg/mL, respectively, which rose to 254.3 ($P < 0.01$), 236.1 ($P < 0.01$), and 227.2 pg/mL ($P < 0.01$), respectively, after day 5, day 7, and day 14.

TNF-α levels

The TNF-α levels in the muscle, skin, colon, and small intestine wounds measured 1, 3, 5, 7, and 14 days after surgery are presented in Figure 2. In muscle tissue, TNF-α levels were 44.7 pg/mL, which was maximally induced 1 day after the surgery. No significant change in the levels were observed 3 (48.2 pg/mL), 5 (48.8 pg/mL), 7 (50 pg/mL), and 14 days (43.3 pg/mL) post wounding. In skin tissue, TNF-α levels at 1, 3, 5, 7, and 14 days postsurgery were 31.7, 34.2, 46.5, 43.4, and 37.6 pg/mL, respectively. The TNF-α levels on day 5 were significantly higher than those measured on day 1 (46.5 pg/mL vs 31.7 pg/mL, $P < 0.05$), whereas the levels on day 3, 7, and 14 were comparable to those found on day 1. In colon, compared with the values on day 1, TNF-α levels were significantly elevated 3 days after the wounding (26.6 pg/mL vs 18.6 pg/mL, $P < 0.05$) and further peaked after 5 (46.9 pg/mL vs 18.6 pg/mL, $P < 0.01$), 7 (37.8 pg/mL vs 18.6 pg/mL, $P < 0.01$), and 14 days (37.9 pg/mL vs 18.6 pg/mL, $P < 0.01$). In small intestine, TNF-α levels on day 1 (25.3 pg/mL) were found to be significantly increased on day 5 (25.3 pg/mL vs 45.7 pg/mL, $P < 0.01$), 7 (25.3 pg/mL vs 42.3 pg/mL, $P < 0.01$), and day 14 (25.3 pg/mL vs 40.7 pg/mL, $P < 0.01$) after the

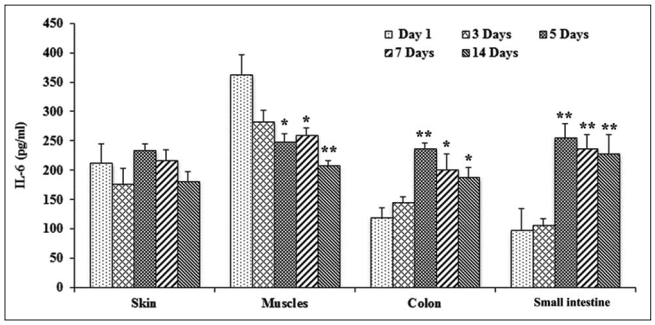

Figure 1: IL-6 levels in skin, muscle, colon, and small intestine tissue wounds. Tissue samples were collected 1, 3, 5, 7, and 14 days after the anastomatic surgery in rats. The IL-6 levels in tissue homogenates were measured using ELISA-based assays. *$P < 0.05$, **$P < 0.01$

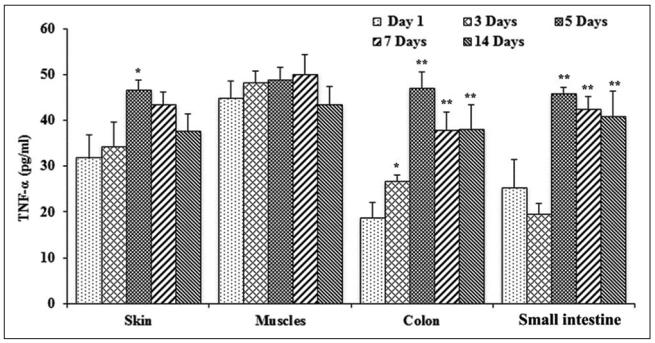

Figure 2: TNF-α levels in skin, muscle, colon, and small intestine tissue wounds. Tissue samples were collected 1, 3, 5, 7, and 14 days after the anastomatic surgery in rats. The TNF-α levels in tissue homogenates were measured using ELISA-based assays. *$P < 0.05$, **$P < 0.01$

surgery. No change in TNF-α levels were found on day 3 (19.4 pg/mL) compared with day 1 (25.3 pg/mL).

IFN-γ

The levels of IFN-γ in skin, muscle, colon, and small intestine colonic wounds are presented in Figure 3. In muscle tissue, the IFN-γ levels were maximally induced 1 day after the surgery (157.3 pg/mL) and no significant change in the levels was observed 3 (159.8 pg/mL), 5 (171.3 pg/mL), 7 (163.1 pg/mL), and 14 days (154.5 pg/mL) post wounding. In skin tissue, IFN-γ levels at 1, 3, 5, 7, and 14 days postsurgery were 110.5, 109.4, 157.8, 134.4, and 126.2 pg/mL, respectively. The IFN-γ levels on day 5 were significantly higher than those measured on day 1 (157.8 pg/mL vs 110.5 pg/mL, $P < 0.05$), whereas the levels on day 3, 7, and 14 were comparable to those found on day 1. In colon compared with day 1 (78.3 pg/mL) and day 3 (91.5 pg/mL), the IFN-γ levels were significantly increased 5 (78.3 pg/mL vs 175.6 pg/mL, $P < 0.01$), 7 (78.3 pg/mL vs 133.2 pg/mL, $P < 0.01$), and 14 days (78.3 pg/mL vs 153.6 pg/mL, $P < 0.01$) post wounding, whereas no significant change in the IFN-γ levels were noted between day 1 (78.3 pg/mL) and day 3 (91.5 pg/mL) after the wounding. In the small intestine, compared with levels at day 1 (55.4 pg/mL), IFN-γ levels significantly increased, peaking at day 5 (170.3 pg/mL vs 55.4 pg/mL, $P < 0.001$) and with significant increases at 7 (165.5 pg/mL vs 55.4 pg/mL, $P < 0.001$), and 14 (166.8 pg/mL vs. 55.4 pg/mL, $P < 0.001$) days after the surgery. IFN-γ levels on day 1 (55.4 pg/mL) and day 3 (65.4 pg/mL) were comparable.

DISCUSSION

In the present study, we compared the timecourse of IL-6, TNF-α, and IFN-γ expression in rat skin, muscle, small intestine, and colon tissue, after anastomosis, to understand the differences in the kinetics of their expression at wound sites, and correlations with the wound healing rates of these tissues. The expression of these inflammatory cytokines was measured after 1, 3, 5, 7, and 14 days of anastomosis. Among the studied wounds, we found the highest increase in IL-6 levels in the muscle 1 day after the surgery with the levels remaining elevated on day 3 and followed by a significant drop on day 5. No further decrease was noted on days 7 and 14. In the colon and small intestine, the pattern of IL-6 expression was identical, with low IL-6 levels on days 1 and 3 followed by a significant increase 5 days postwounding and the increased levels were sustained up to days 7 and 14. Although in the skin the IL-6 levels remained low and constant throughout the study period. Previous studies that have examined the effect of IL-6 on healing process have been ambiguous, with a study indicating a deleterious effect of IL-6 on colon wound healing, which has been shown to prolong the healing process,[16] whereas other studies have demonstrated the favorable role of IL-6 in skin wound healing, which accelerated the healing process.[22,23] In human breast cancer patients, the IL-6 levels exhibited a significant jump 1 day after the mastectomy,[24] which is consistent with our finding where a maximum IL-6 expression was noted 1 day postsurgery. IL-6 has also been recently shown to be upregulated in cardiac muscle wound healing through the downregulation of fibrosis in cardiac muscle fibroblasts.[25] The highest levels of serum IL-6 were reported in children undergoing cardiac surgery.[26] Similarly, IL-6 has been shown to be elevated and exert a role in activating STAT3 signaling to improve healing of muscle injury.[27] Tissue transglutaminase (TG), which stabilizes the structure of tissues by covalently ligating extracellular matrix molecules, is linked to muscle wound healing, and it incidentally is correlated with a concomitant increase in IL-6 levels.[28] Above data clearly establishes the pivotal role of IL-6 in wound healing. The increase in IL-6 levels 5, 7, and 14 days after the surgery may contribute to the faster healing of intestinal and colonic wounds as compared to increased IL-6 levels on days 1 and 3 followed by drop after days 5, 7, and 14 in the muscle. Importantly, IL-6 also acts as an anti-inflammatory molecule in addition to its well-known pro-inflammatory function and therefore its sustained elevation up to day 5 after surgery in colon and small intestine may have likely contributed to faster wound healing by limiting the expression of other pro-inflammatory cytokines. On the other hand, IL-6 is unlikely to have a role in skin wound healing owing to a lack of change in its levels throughout the study duration or possibly, the levels increased even before day 1, which is the earliest measured duration in the study, which points to the importance of sustained IL-6 increase in skin wound healing.

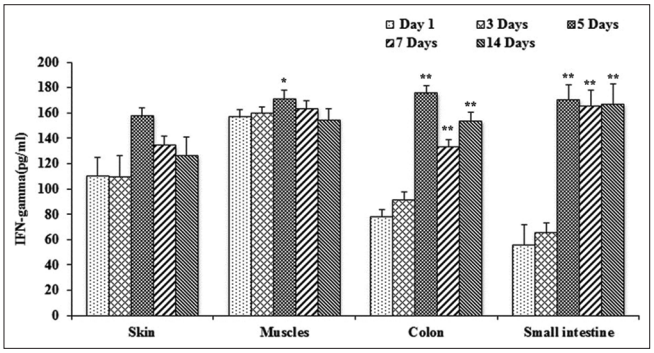

Figure 3: IFN-γ levels in skin, muscle, colon, and small intestine tissue wounds. Tissue samples were collected 1, 3, 5, 7, and 14 days after the anastomatic surgery in rats. The IFN-γ levels in tissue homogenates were measured using ELISA-based assays. *$P < 0.05$, **$P < 0.01$

The IFN-γ has also been shown to affect wound healing. Previous studies have shown that systemic administration of IFN-γ impairs wound healing presumably through modulating collagen deposition and neovascularization in the wound site.[29] Similar results were obtained after the

IFN-γ administration in a skin wound model where IFN-γ delayed the healing process.[30] In myofibroblasts, which have a role in the production of granulated tissue, IFN-γ reduced wound contraction.[31] Increased expression of IFN-γ is also reported in the radiation-induced skin wounds.[32] In myofibroblasts, which have a role in the production of granulated tissue, IFN-γ reduced wound contraction.[31] IFN-γ knockout (KO) mice exhibited an accelerated wound healing evidenced by rapid wound closure and granulation tissue formation.[13] Moreover, IFN-γ KO mice exhibited enhanced angiogenesis with augmented vascular endothelial growth factor mRNA expression in wound sites, compared with wild-type mice. IFN-γ KO mice also exhibited accelerated collagen deposition with enhanced production of TGF-β1 protein in wound sites, compared with wild-type mice. A recent study demonstrated the biphasic temporal expression of IFN-γ from 3 or 6 h to 12 h or 1 day and from 3 or 5 days to 7 days in skin burn injuries.[6] Collectively, the above studies indicate that IFN-γ negatively influences wound healing. In our study, we found a constant and maximal expression of IFN-γ in muscle wound throughout the study duration, while no significant change in its levels was found at any of the examined time. Whereas in skin wounds, IFN-γ levels remained low 1 and 3 days after the surgery and peaked on day 5 and thereafter reduced on day 7 and 14. We found a similar trend of IFN-γ expression in colon and small intestine with the lowest levels on days 1 and 3 and peaking on day 5, thereafter the cytokine levels remained elevated at least up to 14 days postwounding. Thus, given the negative role of IFN-γ in wound healing, its sustained increase in the colon and intestinal wounds deviates from the previously reported observations about the faster healing of these wounds. It is likely that effect of IFN-γ on the healing of colon and small intestinal wounds are masked by cytokine/s, which is not examined in the present study. Lower levels of IFN-γ in skin found in our study also are against the reported delayed healing of skin wounds, whereas the sustained and maximal induction of IFN-γ in the muscle are consistent with the prolonged healing in this tissue.

The colonic wound fluids have shown to have increased TNF-α levels as compared to breast tissue wounds, which showed a decreased expression of components purported to have positive role in wound healing.[33] TNF-α expression increased about threefold in colonic wounds treated with LPS over that in the control group 1 h after the anastomosis, whereas TNF-α-reactive cells were observed until 24 h after the operation in the LPS group, thus local expression of TNF-α at the anastomotic site suggests an inhibitory factor in the wound healing process.[18] In human dermal fibroblasts, TNF-α suppresses alpha-smooth muscle actin expression and the mechanical testing showed that TNF-α decreases the stiffness and contraction of the lattices changes in collagen, and fibronectin expression resulting in abnormal and delayed wound healing.[34] In contrast, TNF-α inhibition throughout healing has shown to qualitatively impair wound healing.[10] In human patients with intestinal wounds, a steady increase in wound fluid content of TNF-α was noted.[35] Thus TNF-α has been shown to both enhance and attenuate wound healing; however, its effect likely depends on the kinetics of its expression. In the present study, there was an early and significant increase in TNF-α levels in the colon (day 3) and small intestine (day 5) wounds; moreover, these levels persisted 5, 7, and 14 days after the surgery. In contrast, TNF-α levels peaked 5 days postwounding in the skin and the levels dropped to those found on day 1. In muscle, the TNF-α levels demonstrated constant elevation starting from day 3 to day 14.

CONCLUSION

We found a unique, consistent pattern of kinetics of IFN-γ, TNF-α, and IL-6 expression in the small intestinal and colonic anastomotic wound during healing, which differed from the expression kinetics of these cytokines in the skin and muscle. This difference in the timecourse of cytokine expression may presumably play a crucial role in the faster healing of small intestinal and colonic wounds.

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