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Critical role of tumor necrosis factor-α in the early process of wound healing in skin

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Abstract

Tumor necrosis factor (TNF)-α is quickly released and initiates inflammation at wound tissues, but its precise role in wound healing is not fully understood. We examined the contribution of this cytokine to the early process of healing using a mouse model with full-thickness wounds in skin. TNF-α synthesis was detected just after wound creation, increased during the first several hours, reached a peak level on day 1, and then decreased to the basal level. In mice treated with anti-TNF-α mAb, wound closure was significantly delayed, and distances between the panniculus carnosus edges were significantly longer on day 3, compared with control. Inflammatory cell and fibroblast density were markedly decreased on day 3 in the anti-TNF-α mAb-treated mice compared with control. In contrast, wound healing was accelerated on day 3 when mice were treated with bioactive TNF-α. These results indicate that TNF-α is involved in the early process of wound healing.

Keywords: Wound healing; Skin; TNF-α; Anti-TNF-α mAb

1. Introduction

Skin has the capacity to repair damaged or lost tissues by regenerating tissues and forming a collagenous scar. This process is called "wound healing" (Robson et al., 2001) and consists of coagulation, inflammation, proliferation and remodeling phases, in which a variety of cytokines and growth factors, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) are produced (Rumalla and Borah, 2001; Mast and Schultz, 1996; Barrentos et al., 2008). Both immune

Abbreviations: TNF-α, tumor necrosis factor-α; mAb, monoclonal antibodies; ELISA, enzyme-linked immunosorbent assay; HE, hematoxylin-eosin; SD, standard deviation.

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and non-immune cells, including macrophages, neutrophils, fibroblasts, vascular endothelial cells and keratinocytes, are involved in this process. Among these cytokines, TNF-α is quickly released by vascular endothelial cells, keratinocytes and fibroblasts in the injured area, and this initiates the inflammatory phase by promoting inflammatory leukocyte recruitment into the wounded tissues (Mast and Schultz, 1996). In the inflammatory phase, major cellular sources of TNF-α are changed into recruited neutrophils and macrophages, and this process yields a positive amplification circuit for extending the inflammatory responses (Mast and Schultz, 1996; Barrentos et al., 2008; Eming et al., 2007). In addition, TNF-α regulates the activity of fibroblasts, vascular endothelial cells and keratinocytes, and the synthesis of extracellular matrix proteins and matrix metalloproteinases, which are closely involved in the healing of injured tissues (Mast and Schultz, 1996).

In the present study, we investigated the effect of suppressing TNF-α biological activity on the healing process of full thickness wounds created in the skin of mice. TNF-α biological activity was suppressed by neutralizing monoclonal antibodies (mAb). In addition, we also tested how this process was affected by administering bioactive TNF-α. We found that TNF-α played an important role in the early process of wound healing in the skin.

2. Materials and methods

2.1. Animals

ICR mice were obtained from Clea Japan, Inc. (Tokyo, Japan). Mice were subjected to experiments at 7–10 weeks of age. To minimize possible discomfort to animals, m-acetamidophenol (0.4 mg/mouse/day) was administered in the drinking water during the experimental period. All experimental protocols described in the present study were approved by our university's Ethics Review Committee for Animal Experimentation.

2.2. Wound creation and tissue collection

Mice were anesthetized by intraperitoneal injection of 40 mg/kg sodium pentobarbital (NEMBTAL Injection, Dainippon Sumitomo Pharma, Osaka, Japan). The dorsal hairs were shaved to fully expose the skin, which was then rinsed with 70% ethanol. Six full-thickness wounds extending to the panniculus carnosus were created using a 3-mm diameter biopsy punch (Biopsy Punch, Kai industries Co., Ltd., Gifu, Japan) under sterile conditions. The six wounds per mouse were at least 5 mm apart. The injured areas were covered with a polyurethane film (Tegaderm Transparent Dressing, 3 M Health Care, St. Paul, MN) and an elastic adhesive bandage (Hilate, Iwatsuki, Tokyo, Japan) as an occlusive dressing. At various time points, mice were sacrificed and the wound tissues were collected by excising the area with an 8-mm diameter biopsy punch. The tissues were processed for histological analysis and measurement of cytokine concentrations.

2.3. Reagents and antibodies

Anti-TNF-α mAb was purified using a protein G column kit (Kirkegaard & Perry Lab., Gaithersburg, MD) from the culture supernatants of hybridoma cells (clone MP6-XT2.2-11, provided by Dr. Akio Nakane, Department of Microbiology and Immunology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan). To neutralize TNF-α biological activity, mice were injected intraperitoneally with mAb against this cytokine at 400 μg one day prior to and three days after wound creation. Rat IgG (ICN Pharmaceuticals, Inc., Aurora, OH) was used as the control Ab. An amount of 25 μg/kg TNF-α, purified from a human B-cell lymphoblastoid cell line (BALL-1, Hayashibara Biochemical Laboratories Inc., Okayama, Japan), was injected into the peritoneal cavity once daily beginning on the day of wound creation (Fukuda et al., 1998). PBS was used as the control vehicle.

2.4. Measurement of wound area

Morphometric analysis was performed on digital images obtained using a digital camera (QV-2800UX, Casio, Tokyo, Japan). After the wounds were created, photographs were taken of each wound before dressing. At various time points, the polyurethane films were gently removed from the experimental mice, and the wounds were photographed. The wound area was quantified by tracing its margin and calculating the pixel area using AxioVision imaging software Release 4.6 (Carl Zeiss Micro Imaging Japan, Tokyo, Japan). Wound size at any given time point after the initial wound creation was expressed as a percentage of the original wound area.

2.5. Measurement of cytokine concentrations

Wound tissues were homogenized in PBS, and the TNF-α concentration in the supernatant was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Cambridge, MA). The results were expressed as the values per one wound. The detection limit was 5.1 pg/ml.

2.6. Histological analysis

Wound tissues were collected using an 8-mm diameter biopsy punch and were dissected in a caudocranial direction. The tissues were fixed with 4% paraformaldehyde-phosphate buffer solution and embedded in paraffin. Sections 2 μm in thickness were harvested from the central portion of the wound and stained with hematoxylin-eosin (HE), according to the standard method. The distance between the edges of the panniculus carnosus was measured to determine the degree of wound contraction on the HE-stained paraffin section.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis between groups was performed using the Student's *t* test. A *p* value less than 0.05 was considered significant.

3. Results

3.1. Skin wound healing and local TNF-α production

For the animal model of skin wound healing, we created full thickness wounds in the backs of mice and observed the condition of the wounds at various time intervals. On macroscopic observation, the wounds showed steady closure in a time-dependent manner, decreasing by 80% in size by day 14, without eschars, exudative fluids and any signs of infection (Fig. 1A and B). We next measured the TNF-α concentration in the homogenate supernatants of the collected wounds at various time intervals. As shown in Fig. 2, TNF-α was detected just after wound creation, and rapidly increased in concentration during the first several hours. The peak concentration level was reached on day 1, and levels declined thereafter to the basal level.

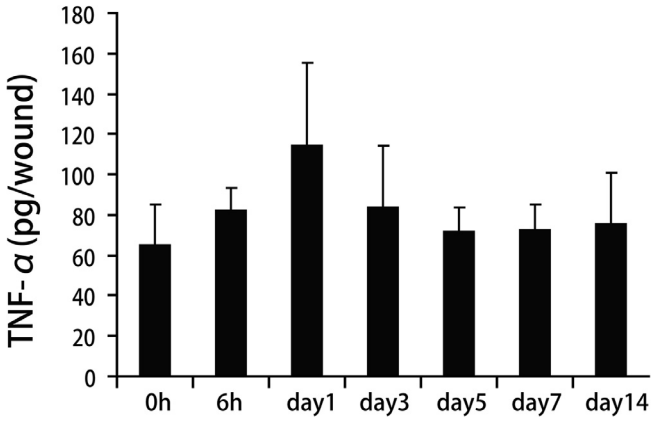

Figure 2. TNF-α concentration in wounded tissue homogenate TNF-α concentration in the wound tissue homogenate was measured at 0 and 6 h and on days 1, 3, 5, 7 and 14 after wound creation. Each column represents the mean ± SD of 3 homogenates, each of which was prepared from 6 wounds.

3.2. Effect of anti-TNF-α mAb on wound healing

To address the role of TNF-α in wound healing, we examined the effect of neutralizing anti-TNF-α mAb on this process. As shown in Fig. 3A, in mice treated with

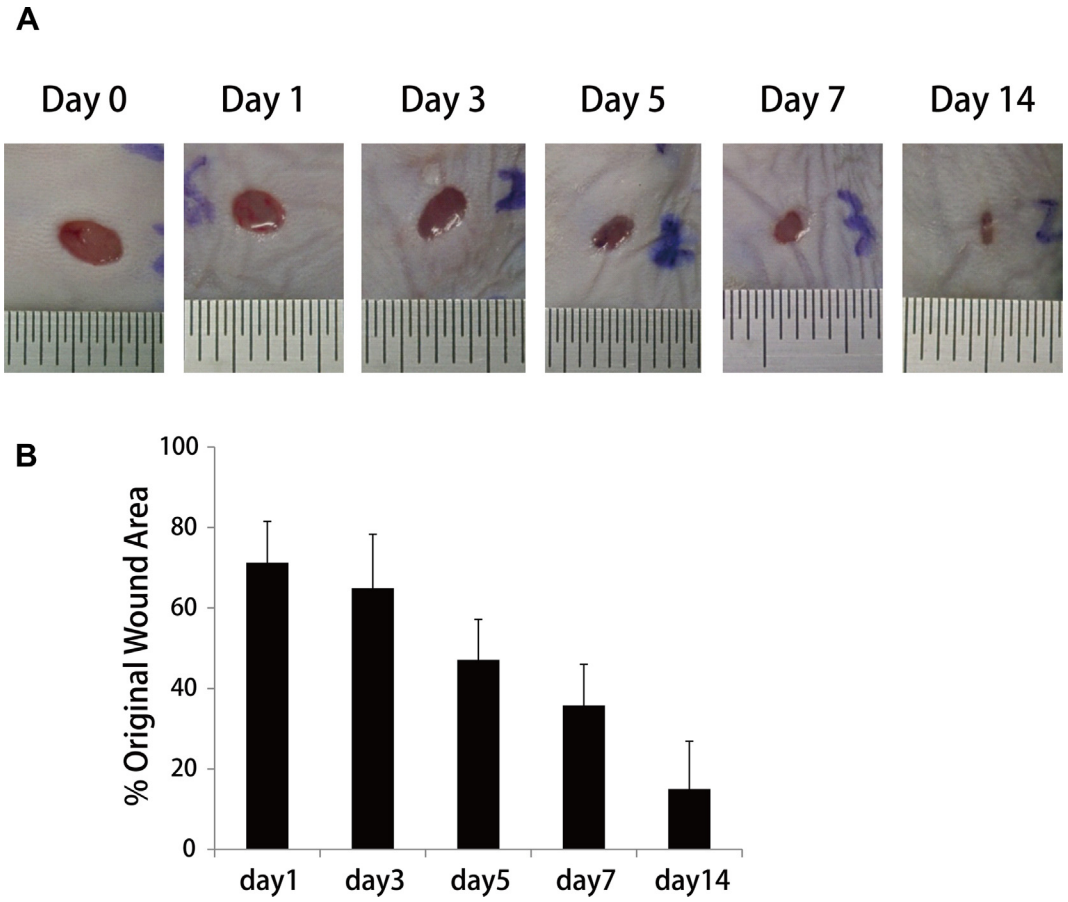

Figure 1. A mouse model of wound healing Wounds were created in the backs of ICR mice using biopsy punches. Wounds were observed on days 1, 3, 5, 7 and 14 after wound creation. The images (A) and percentages of original wound area (B) are shown for each day. Each column represents the mean ± SD of 24 wounds.

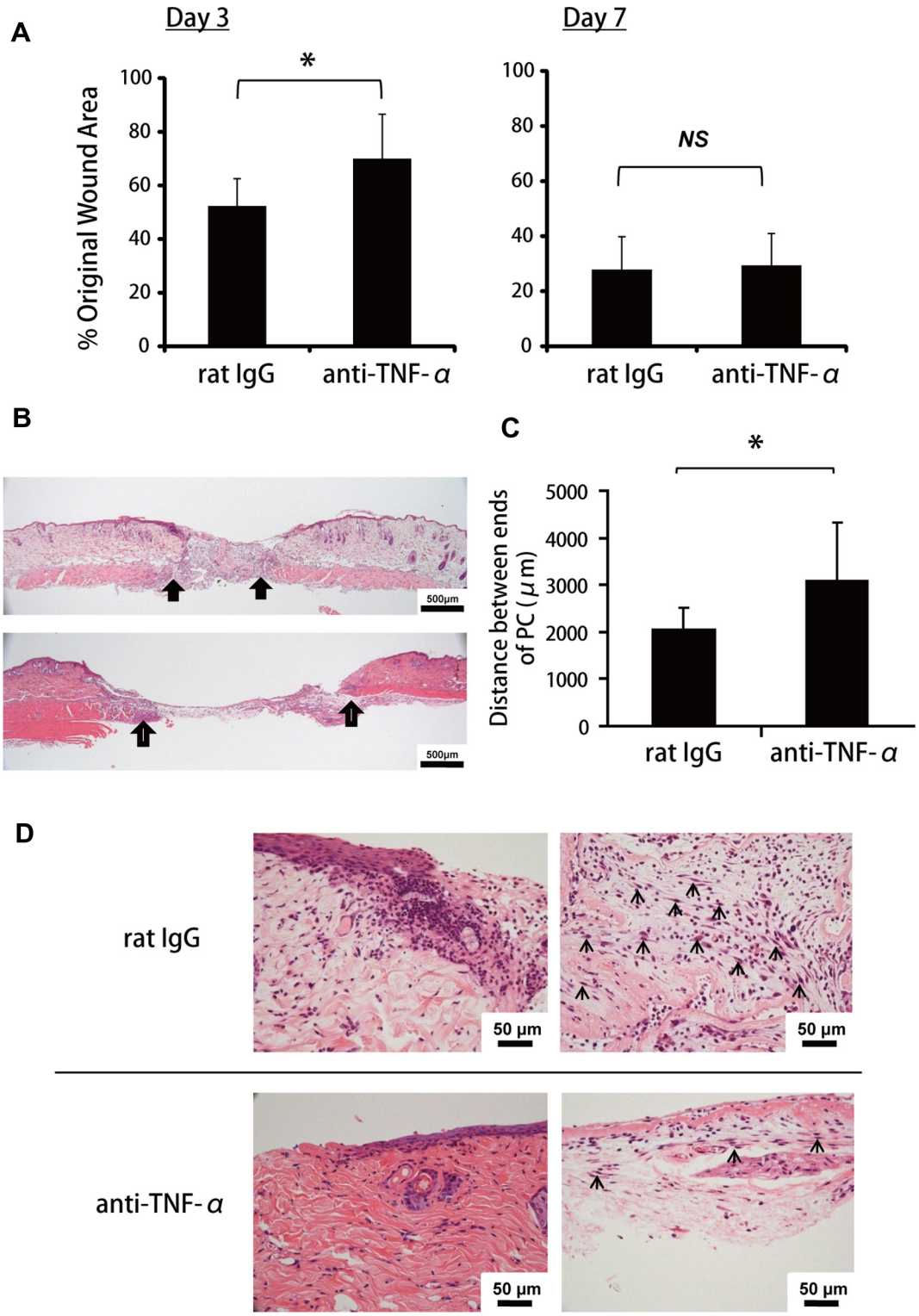

Figure 3. Effect of anti-TNF-α mAb treatment on wound healing Mice were treated with anti-TNF-α mAb (400 μg/mouse) or control IgG (400 μg/mouse). (A) The wound area size was evaluated on days 3 and 7. Each column represents the mean ± SD of 18 to 24 wounds. *NS*, not significant; $^{*}p < 0.05$. (B, C) The distance between the edges of the panniculus carnosus on the HE stained paraffin sections was evaluated on day 3. Each column represents the mean ± SD of 8–10 sections. Arrows indicate the edges of panniculus carnosus. PC, panniculus carnosus; $^{*}p < 0.05$. (D) Inflammatory cell and fibroblast density on the HE stained paraffin section was markedly decreased in the anti-TNF-α mAb-treated mice compared with control mice on day 3. Arrows indicate fibroblasts.

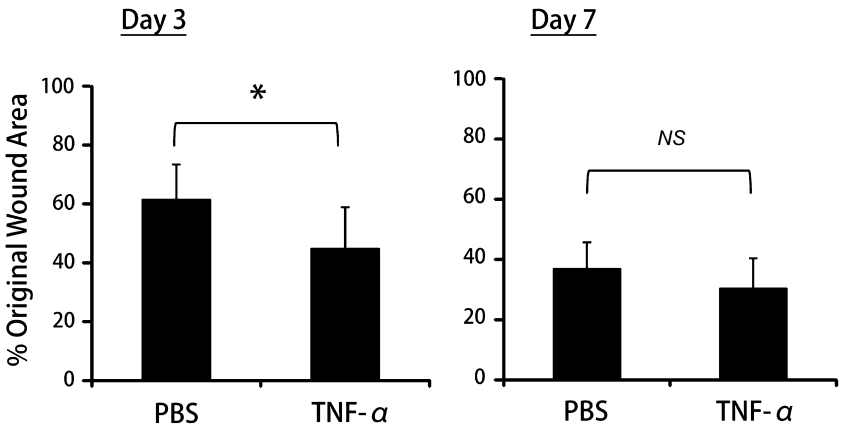

Figure 4. Effect of TNF-α administration on wound healing. Mice were treated with purified TNF-α (25 μg/kg) or PBS, and wound areas were evaluated on days 3 and 7. Each column represents the mean ± SD of 18–24 wounds. *NS*, not significant; $^{*}p < 0.05$.

anti-TNF-α mAb, wound closure was significantly delayed on day 3 compared with that in control mice treated with rat IgG, although the size of the original wound area was not significantly different between both groups on day 7. These macroscopic findings were confirmed by histological assessment. As shown in Fig. 3B and C, HE-stained paraffin sections representing the longitudinal diameter of the wound showed significantly shorter distances between the edges of the panniculus carnosus in treated mice compared with control animals on day 3. In addition, on day 3 after wound creation in the anti-TNF-α mAb-treated mice, the density of inflammatory cells and fibroblasts was markedly decreased when compared with control mice (Fig. 3D).

3.3. Effect of TNF-α administration on wound healing

To further elucidate the role of TNF-α, we tested whether administration of this cytokine affected the wound healing process. As shown in Fig. 4, this treatment resulted in a significant promotion of wound closure compared with PBS treatment on day 3, but not on day 7.

4. Discussion

The major findings in the present study are as follows: (1) TNF-α production was detected in the homogenate of wound tissues with a peak level on day 3 after wound creation; (2) wound healing was significantly delayed on day 3, but not on day 7, in mice treated with anti-TNF-α mAb compared with mice that received control IgG; and (3) TNF-α accelerated wound repair on day 3, but not on day 7. These results indicate that TNF-α is closely involved in the very early process of wound healing in skin.

TNF-α was produced in the wounded tissues and reached a peak level on day 1, after which it returned to the basal level. However, this increase in TNF-α level was not statistically significant even at the time point of maximal production. This may be a result of the high TNF-α level detected just after wounding (0 h), which may make the relatively small production of this cytokine less clear. In our unpublished data, TNF-α was constitutively detected in the skin tissues taken from mice that did not undergo any treatment. Also, in previous studies, pro-inflammatory cytokines including TNF-α were detected at the mRNA level using RT-PCR, particularly in epidermal cells, using in-situ hybridization (Sato and Ohshima, 2000; Takacs et al., 1998). This constitutive expression of TNF-α is thought to be involved in the regulation of homeostasis in skin tissue (Mckay and Leigh, 1991). Thus, TNF-α levels measured in the wound tissues were likely higher than the normal basal levels, which suggests that this cytokine may be involved in the process of wound healing.

In support of this hypothesis, treatment with neutralizing anti-TNF-α mAb resulted in a delay in wound closure and administration of bioactive TNF-α resulted in promotion of wound closure, particularly during the early phase. In addition, closure of the panniculus carnosus was delayed by treating the wounded mice with neutralizing anti-TNF-α mAb. During the proliferation phase in the wound healing process, TNF-α secreted from macrophages promotes the formation of the extracellular matrix in the wounded tissues by inducing the generation of proteoglycan and fibronectin by fibroblasts. This process is further enhanced by collagen synthesis from the proliferating fibroblasts, which are promoted by TGF-β. Thus, proteases secreted by macrophages and fibroblasts are closely involved in the generation of bioactive TGF-β from pro-TGF-β, which is produced by fibroblasts (Witte and Barbul, 1997). On the other hand, it is well known that TGF-β1 and PDGF produced by macrophages stimulate the generation of myofibroblasts from some fibroblasts, which results in the contraction of the extracellular matrix and subsequent wound closure (Desmouliere et al., 1993; Yang et al., 1997). These earlier findings and those of our present study suggest that neutralization of bioactive TNF-α may delay closure of the panniculus carnosus and overall wound healing by interfering with the proliferation

of fibroblasts and the formation of the extracellular matrix created by these cells.

In the present study, the cellular source of TNF-α production remains to be determined. Because TNF-α was detected quickly after wound creation, non-immune cells, such as keratinocytes and fibroblasts, and innate immune cells, such as neutrophils and macrophages, may contribute to synthesis of this cytokine. Further investigation is necessary to address this issue.

5. Conclusion

We demonstrated that TNF-α plays a crucial role in the early process of wound healing in skin and that TNF-α administration had a beneficial effect on this response. Thus, the current finding may lead to development of a novel therapeutic strategy for chronic skin wounds, such as intractable decubitus, in which the wound healing process is impaired.

Conflict of interest

None.

Acknowledgements

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