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Interleukin-6 Treatment Augments Cutaneous Wound Healing in Immunosuppressed Mice

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ABSTRACT

It has been postulated that the inflammatory response that occurs after cutaneous wounding is a prerequisite for healing and that inflammatory cytokines, such as interleukin-6 (IL-6) are involved in this process. We showed previously that IL-6-deficient mice display delayed wound healing, which could be reversed by administration of a murine IL-6 expression plasmid or recombinant murine IL-6 (rMuIL-6). In the present study, we observed that delayed cutaneous wound healing, which occurs as a result of glucocorticoid-induced immunosuppression, can also be reversed by rMuIL-6, as evidenced by epithelialization, granulation tissue formation, and wound closure. In vehicle control mice, rMuIL-6 did not augment healing but rather delayed the process. Immunochemical studies indicated that the expression of matrix metalloproteinase-10 (MMP-10) was increased in dexamethasone-treated mice and that rMuIL-6 treatment reduced its expression, indicating that IL-6 may influence dermal matrix formation and, specifically, collagen synthesis. These results demonstrate that IL-6 can restore abnormal wound repair that occurs in immunodeficiency and suggest its use as a potential therapy.

INTRODUCTION

CUTANEOUS WOUND REPAIR IN MOST MAMMALS follows an orderly sequence of events that are well defined and have been thoroughly reviewed elsewhere.[1] The role of the inflammatory response, which precedes wound healing, is thought to be primarily related to stemming infection and removal of cellular debris. However, increasing evidence suggests that inflammatory cytokines may also be involved in modulating the healing process, and a number of studies indicate that anti-inflammatory agents, such as glucocorticoids, significantly delay cutaneous wound healing.[2,3] Glucocorticoids, directly or indirectly, influence many processes associated with cutaneous wound repair, including matrix metalloproteinase (MMP) expression, bacterial clearance, neovascularization, collagen deposition, and expression of proinflammatory cytokines.[4] The anti-inflammatory activity of glucocorticoid, however, is thought to be the major cause of delayed repair, suggested by the fact that glucocorticoids are effective at delaying wound repair only if given before the inflammatory response is initiated.[3]

Among such proinflammatory cytokines, interleukin-6 (IL-6) is a major mediator of the host response to tissue injury[5,6] and is present in wound fluid from mice and humans.[7–10] We recently reported that IL-6-deficient or immunosuppressed mice demonstrate impaired wound healing, and this could be prevented by administration of recombinant IL-6 or intradermal injection of an expression plasmid containing the full length murine IL-6 (MuIL-6) cDNA.[11] Consistent with these observations, *in situ* hybridization of wound tissue from wild-type mice demonstrated that IL-6 mRNA is expressed at high levels in epidermal keratinocytes at the leading edge of the wound. Although IL-6 is not produced spontaneously in intact normal skin, it is induced in the skin in response to bacterial endotoxins, skin irritants, contact allergens, viruses, UV radiation, and thermal damage, suggesting a broad role in dermatotoxic reac-

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tions.[12–15] IL-6 is also associated with a number of chronic pathologic skin conditions, including psoriasis,[16] scleroderma,[17] and systemic lupus erythematosus (SLE).[18]

The function of IL-6 in wound healing has not been clearly established. Whereas IL-6 induces keratinocyte proliferation in culture,[16] it does not influence mitogenesis *in vivo*.[19] Furthermore, keratinocyte proliferation is not enhanced in transgenic mice that overexpress dermal IL-6, although a thickened stratum corneum is present.[20] IL-6 may assist wound healing indirectly by modulation of growth factors or their receptors. In this respect, IL-6 induces keratinocyte growth factor (KGF) expression in fibroblasts[21,22] and epidermal growth factor (EGF) receptors in cultured keratinocytes.[23] When overexpressed in normal rat skin, IL-6 induces transforming growth factor-α (TGF-α),[24] a potent keratinocyte mitogen, which, in turn, induces IL-6, indicating a paracrine interaction.[25] IL-6 also acts in a protective manner by decreasing connective tissue matrix degradation through inhibition of superoxide anion production associated with chronic inflammation[26] and by increasing tissue inhibitors of matrix metalloproteinase (TIMP) expression.[27–31]

The amelioration of chronic wounds, such as those associated with diabetes or immunosuppression, has been an elusive goal. Chronic wounds associated with diabetes and glucocorticoid treatment appear to share several characteristics, such as altered expression of cytokines and increased activity of MMP. MMP, such as collagenase I (MMP-1) or stromelysin 2 (MMP-10), are upregulated during normal wound healing[32] and are overexpressed in the wounds of dexamethasone-treated mice.[3] The increased activities of these proteinases are thought to delay wound healing by inhibiting collagen matrix deposition and tissue remodeling.[3] Another characteristic of chronic wounds is decreased levels of certain cytokines. Expression of such proinflammatory cytokines as tumor necrosis factor-α (TNF-α), IL-1, and IL-6 is inhibited by glucocorticoid treatment,[3] and IL-6 levels are decreased in wound fluid from diabetic mice, which display elevated corticosterone levels and delayed wound healing.[33] In this report, we expand our previous studies by detailing the augmentation of wound healing in dexamethasone-treated mice by rMuIL-6. Additionally, we present data suggesting that IL-6 mediates its effects by helping to regulate MMP-10 expression.

MATERIALS AND METHODS

Experimental model

Male, wild-type (C57BL/6J) mice (Jackson Laboratories, Bar Harbor, ME), weighing 22–28 g and approximately 8–12 weeks old, were housed in polycarbonate cages containing hardwood chip bedding at 21°C ± 2°C on a 12-h light/dark cycle. Mice were treated in accordance with the criteria outlined in the PHS Policy on Humane Care and Use of Laboratory Animals and the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23) in facilities accredited by AAALAC. Mice were anesthetized by intraperitoneal (i.p.) injection with 80 mg/kg pentobarbital, and the left flank was clipped and swabbed with Betadine® (Purdue Frederick Co., Norwalk, CT) and 70% ethanol three times before wounding. Punch biopsies were performed on the shaved area using a 4-mm sterile disposable biopsy punch. Groups of mice were injected subcutaneously (s.c.) with 1 mg/kg/day dexamethasone (USP) (Sigma Chemical Co., St. Louis, MO) for 7 consecutive days before wounding and each subsequent day after wounding. rMuIL-6 (R&D Inc., Minneapolis, MN) was administered as a single dose s.c. at a concentration of 1 mg/kg. The wounds were photographed using a Contax 167MT equipped with a 50-mm macro lens, 52 mm +4 Vivitar closeup attachment, and Vivitar Auto thyristor 550 FD flash. Wound tissue was collected (2–3-mm border was excised around the wound) at specified time points. Tissues were preserved by flash freezing or placed in buffered formalin for histologic study. Histologic sections were stained with hematoxylin and eosin unless otherwise noted.

Ribonuclease protection assay (RPA)

Quantification of RNA samples was performed using an RPA III kit (Ambion, Austin, TX) according to the manufacturer's instructions. The dsDNA template for proinflammatory cytokines (mCK-3b) was purchased from PharMingen (San Diego, CA). ^{32}P-labeled cRNA probes were produced from dsDNA templates using the MAXIscript T7 kit (Ambion) according to the manufacturer's instructions. Aliquots of total RNA were assayed using the RiboQuant kit (PharMingen), and the samples were electrophoresed on a sequencing gel. Protected fragments were quantified using a PhosphorImaging system and ImageQuant software (Storm 360) (Molecular Dynamics, Sunnyvale, CA).

Immunohistochemistry

Goat polyclonal antibodies to MMP-10 and biotinylated sheep antigoat antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA). Formalin-fixed paraffin-embedded sections were dewaxed, antigen retrieved, and stained for MMP-10 according to instructions supplied by the manufacturer. Neutravidin alkaline phosphatase (Pierce, Rockford, IL) was used as a reporter, and the sections were developed in Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Sigma) for 25 min.

Statistical analysis

Significant differences between groups were determined using multifactor ANOVA. Two factor blocked ANOVA was used, blocking on separate experiments to control for the variation introduced by conducting multiple experiments. When a significant difference was indicated, Fishers LSD and Scheffe's *post hoc* analysis were performed. Statistical analysis was performed using StatView, the statistical package (Abacus Concepts Inc., Berkeley, CA).

RESULTS

Role of glucocorticoid and rMuIL-6 treatment on wound healing

Mice were rendered immunosuppressed by administering 1 mg/kg/day dexamethasone consecutively for 7 days before

wounding and each day thereafter until the animals were sacrificed. This dosing regimen has been shown to inhibit cytokine and growth factor expression following cutaneous wounding.[34] Dexamethasone-treated and vehicle-treated control mice were subjected to full-thickness 4-mm punch biopsies on the left dorsal shaved flank and monitored for up to 11 days. Impairment of wound closure was noted in dexamethasone-treated mice, as evidenced by gross and histologic examination (Fig. 1) and measurements of the wound area (Fig. 2). Dexamethasone-treated mice displayed decreased inflammation, granulation tissue formation, and microvascularization, as well as incomplete reepithelialization (Fig. 1B, E). As shown in Figure 2, a delay in wound closure was noted in dexamethasone-treated mice for as long as 15 days (only up to 11 days shown), nearly triple the healing time seen in wounded control mice ($p < 0.01$ by Dunnett's and two-way blocked ANOVA). Pretreatment of dexamethasone-treated mice with rMuIL-6 significantly enhanced time for wound healing ($p<0.01$ compared with dexamethasone alone), with healing times approaching those of controls. Treatment of mice with rMuIL-6 alone had a slight inhibitory effect on wound healing times (Fig. 2). Histologic examination revealed that wounds from dexamethasone-treated mice reepithelialized and displayed well-formed granulation tissue at a faster rate after IL-6 treatment (Fig. 1C, F) than in those treated only with dexamethasone (Fig. 1B, E).

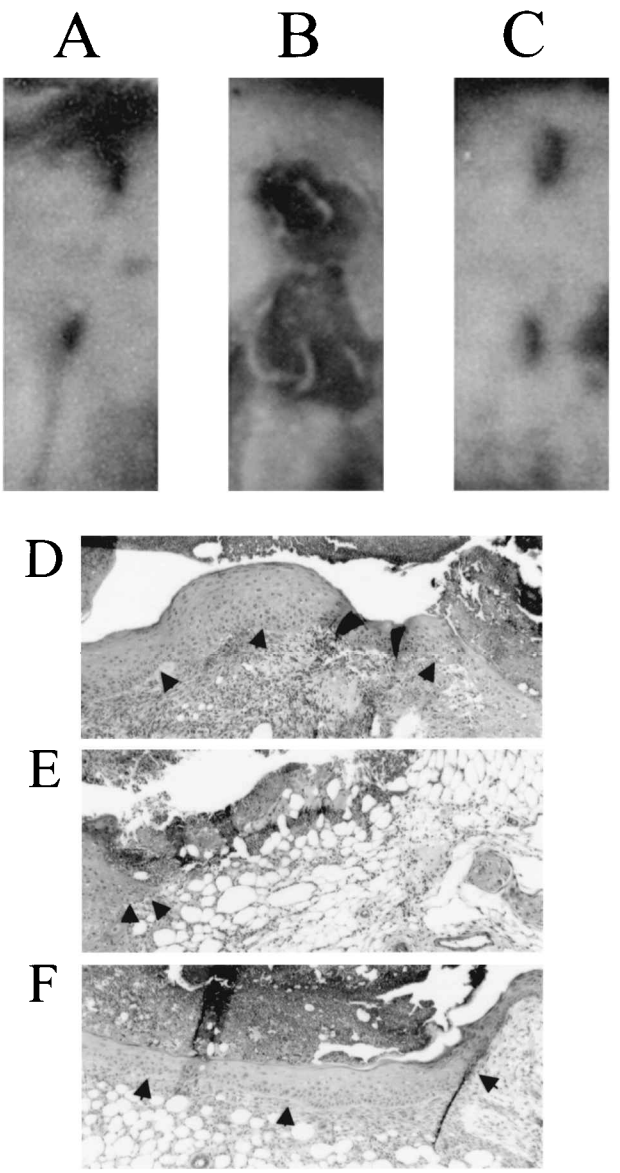

FIG. 1. Wound healing in dexamethasone-treated and IL-6-treated mice. Mice were injected s.c. with 1 mg/kg/day dexamethasone or PBS for 7 days before and 11 days after wounding. Some groups were also administered 1 mg/kg rMuIL-6 s.c. 1 h before wounding. Representative photographs from 5-day-old wounds from (**A**) vehicle control, (**B**) dexamethasone-treated, and (**C**) dexamethasone + rMuIL-6-treated mice. H&E-stained histologic sections (10× magnification) of (**D**) vehicle-treated, (**E**) dexamethasone-treated, and (**F**) dexamethasone + rMuIL-6 pretreated mice. Arrows indicate epithelial layer.

Effects of glucocorticoid treatment on proinflammatory cytokine expression in wound tissue

To determine if dexamethasone treatment inhibited proinflammatory cytokine expression in cutaneous wound tissue, TNF-α and IL-6 mRNA expression was determined by an RPA. RPA revealed that dexamethasone treatment significantly reduced the expression of both TNF-α and IL-6 from skin adjacent to the wound area (Fig. 3). Little, if any, TNF-α or IL-6 was detected in nonwounded skin (Fig. 3).

MMP expression in glucocorticoid-treated mice

Immunohistochemical examination for MMP-10 was performed on formalin-fixed, paraffin-embedded sections of wound tissue collected on days 1–5 after wounding. During this period, wound areas from vehicle-treated animals showed minimal positive staining for MMP-10 (Fig. 4A), whereas wounds from dexamethasone-treated mice revealed robust staining primarily confined to the leading edge of the epithelium (Fig. 4B). Dexamethasone-exposed animals that were pretreated with rMuIL-6 were similar to controls, showing minimum staining for MMP-10 (Fig. 4C).

DISCUSSION

To explore the clinical relevance of our previous studies demonstrating that IL-6 plays a central role in wound healing, we employed a model of wound healing inhibition using glucocorticoid-induced immunosuppression. It is well established that impaired cutaneous healing occurs in individuals receiving glucocorticoid or other immunosuppressive therapies.[2,35] Herein, we show that rMuIL-6 significantly augments wound healing in dexamethasone-treated mice. It should be noted, however, that the wound healing response in immunodeficient mice was not entirely restored by IL-6 treatment, and it is likely that other products cooperate with IL-6 in wound healing responses. This would be expected, considering the broad activ-

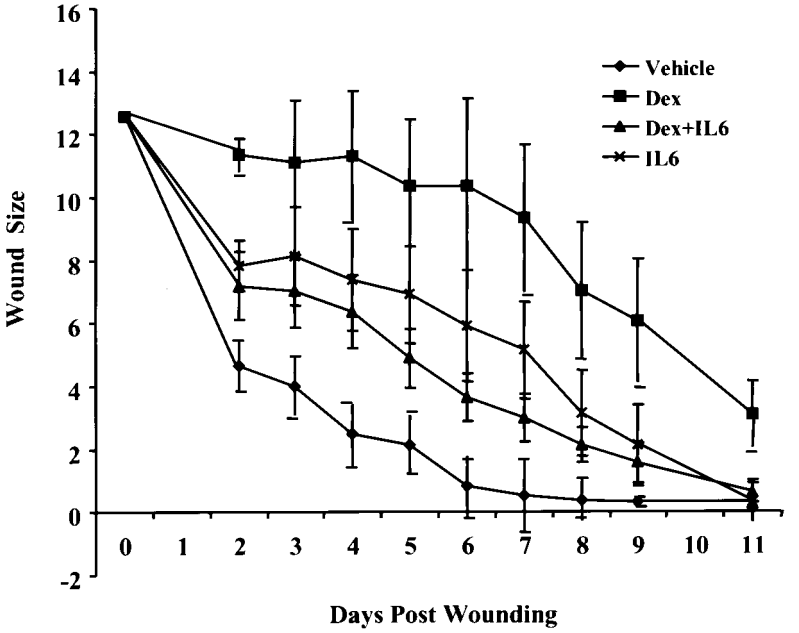

FIG. 2. Time course for wound healing. Mice were injected s.c. with 1 mg/kg/day dexamethasone or vehicle for 7 consecutive days before and 11 days after wounding. Some mice received 1 mg/kg rMuIL-6 1 h before wounding. Mice were wounded as described, and the wounds were photographed at the indicated time points. Photographs of wounds were digitized and analyzed for wound area using NIH image 1.7. The wound area was determined by calculating wound size relative to a 4-mm diameter circular paper cutout placed next to the wound in each photograph. Each data point represents the mean ± SE of the wound area (mm) from 10 mice per group. $n = 10 \pm$ SE; $p < 0.01$ by multifactor ANOVA.

ity of glucocorticoids. For example, it has been shown that the dermal expression of collagen type I,[36] KGF,[34] and TGF-α[37] and most inflammatory cytokines, including IL-6,[3] is inhibited by glucocorticoid treatment. It was also of note that despite a robust IL-6 induction after wounding in immunocompetent individuals,[38] we found that IL-6 administration impairs, rather than augments, wound healing in control wounded mice (Fig. 2). In this respect, it has been reported

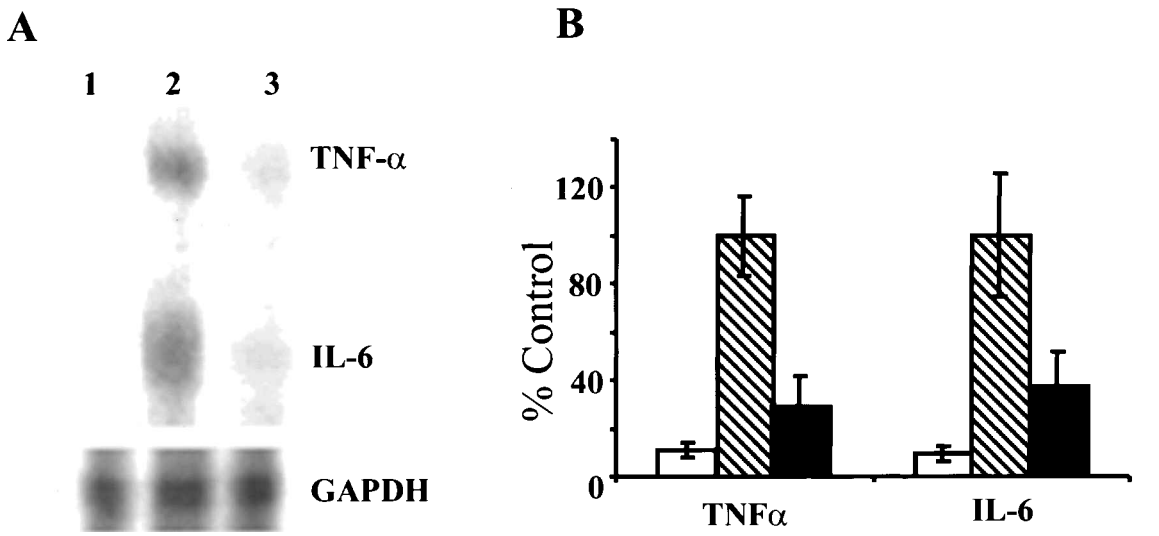

FIG. 3. Dexamethasone decreases proinflammatory cytokine expression in wounded skin. Mice were injected s.c. with either dexamethasone or vehicle as described. Six hours after wounding, the expression of TNF-α and IL-6 was determined by RPA as described. TNF-α expression is shown for comparison purposes. (**A**) Lane 1, unwounded skin; lane 2, wounded control; lane 3, wounded dexamethasone-treated animal. (**B**) Images from PhosphorImager screen were digitized and quantified using NIH image 1.6. Data are expressed as mean percent of control vehicle-treated animals. $n = 3 \pm$ SE.

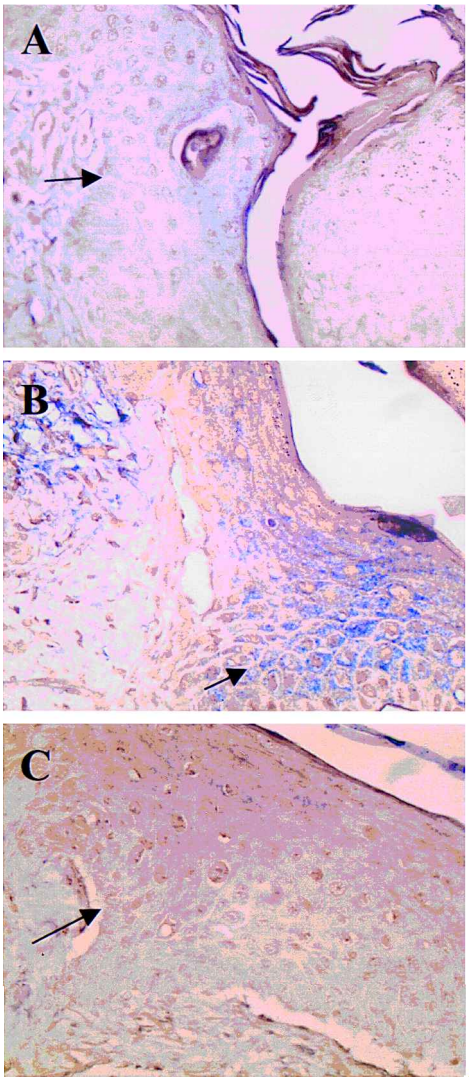

FIG. 4. MMP-10 expression is decreased in dexamethasone-treated mice by rMuIL-6 pretreatment. Sections of wound tissue from treated and untreated mice were taken 1 and 5 days after wounding and stained with biotinylated mouse antibodies to anti-MMP-10 (day 5 shown). (**A**) Control vehicle-treated mouse (40×), (**B**) dexamethasone-treated mouse (20×), and (**C**) dexamethasone + rMuIL-6-treated mouse (40×). Arrows are provided for orientation purposes and indicate interface between the dermis and epidermis.

that pharmacologic doses of TNF-α inhibit wound healing,[39–43] and exogenous IL-6 acts in an anti-inflammatory role in other systems.[44] In immunocompetent animals and humans, a robust IL-6 induction occurs after wounding. It could be that exogenous IL-6 acts in a negative feedback fashion when excess IL-6 is present. Additionally, in a normal wound, the activity of IL-1 or TNF would minimally overlap with that of IL-6,[10] but in our model, IL-6 is given early and is present when these cytokines are induced. When both IL-6 and IL-1 are present at high levels in dermal fibroblast culture, the expression of MMP associated with IL-1 stimulation is augmented.[45,46] This could affect collagen deposition and explain why we observe a delay in wound healing. Thus, it seems that if IL-6 is to be considered as a treatment for impaired wound healing, it may only be efficacious during certain pathologic states in which endogenous IL-6 activity is diminished.

Although increased MMP expression coincides with decreased IL-6 expression and both are associated with delayed wound healing after glucocorticoid administration,[3] as well as experimental autoimmune diabetes,[33] studies on the role of IL-6 in MMP expression show conflicting results.[45,46] IL-6 can increase MMP-1 and MMP-13 expression in rat fibroblasts[47] and is also responsible for MMP-3 induction in skin following UVB exposure.[48] However, IL-6 does not induce MMP in most cell types[30,31,46,49,50] and can display protective activity by producing TIMP in connective tissue cells,[26,27,29,31,46] skin fibroblasts,[30] and alveolar macrophages.[50] This protease is also overexpressed in wounds of dexamethasone-treated mice.[3,51] Herein, we show that rMuIL-6 treatment can suppress MMP-10 expression in the wounds of glucocorticoid-treated mice. MMP-10, also known as stromelysin 2, is normally increased during wound healing[32] and is expressed in the basal keratinocytes at the migratory front of the epidermal wound edge, but not in the dermis.[51] The mechanism by which MMP-10 is upregulated in the wounds of glucocorticoid-treated animals is not known. MMP-10 can be induced in cultured keratinocytes by TNF-α, IL-1β, TGF-β1, KGF, and EGF.[51,52] It is tempting to implicate an indirect mechanism by which IL-6 suppresses these cytokines and growth factors. However, TNF-α, IL-1β, TGF-β1, and KGF are suppressed in the wounds of glucocorticoid-treated mice,[3] and IL-6 upregulates EGF receptors in cultured keratinocytes.[23] Thus, the regulation of these factors does not appear to be related to IL-6 modulation of MMP-10.

When viewed in total, the aforementioned studies indicate that IL-6 may have a dual activity associated with MMP modulation during wound healing. Perhaps during the early inflammatory phase, IL-6 acts in synergy with other cytokines, such as IL-1, to enhance MMP expression, which is necessary for keratinocyte migration and tissue remodeling. During later phases, when proinflammatory cytokine levels have declined, it may act to moderate MMP activity and to protect the formation of the provisional matrix. In this respect, Sato et al.[30] demonstrated that IL-6 can induce only TIMP expression in human fibroblast cultures, whereas when combined with IL-1, it enhances MMP expression. This dual character of IL-6 seems to make it an attractive candidate for wound healing therapy. However, the role of IL-6 in wound healing is obviously very complex, and further investigation is warranted to determine the exact nature of its mechanism before considering it a viable treatment.

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