Impaired cutaneous wound healing in interleukin-6–deficient and immunosuppressed mice

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ABSTRACT **It has been postulated that an inflammatory response after cutaneous wounding is a prerequisite for healing, and inflammatory cytokines, such as interleukin-6 (IL-6), might be intimately involved in this process. IL-6–deficient transgenic mice (IL-6 KO) displayed significantly delayed cutaneous wound healing compared with wild-type control animals, requiring up to threefold longer to heal. This was characterized by minimal epithelial bridge formation, decreased inflammation, and granulation tissue formation. Using electrophoretic mobility shift assays of wound tissue from IL-6 KO mice, decreased AP-1 transcription factor activation was shown compared with wild-type mice 16 h after wounding. *In situ* hybridization of wound tissue from wild-type mice revealed IL-6 mRNA expression primarily in the epidermis at the leading edge of the wound. Delayed wound healing in IL-6 KO mice was reversed with a single dose of recombinant murine IL-6 or intradermal injection of an expression plasmid containing the full-length murine IL-6 cDNA. Treatment with rmIL-6 also reconstituted wound healing in dexamethasone-treated immunosuppressed mice. The results of this study may indicate a potential use for IL-6 therapeutically where cutaneous wound healing is impaired.—Gallucci, R. M., Simeonova, P. P., Matheson, J. M., Kommineni, C., Guriel, J. L., Sugawara, T., and Luster, M. I. Impaired cutaneous wound healing in interleukin-6–deficient and immunosuppressed mice. *FASEB J.* 14, 2525–2531 (2000)**

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For 1995 the U.S. Bureau of Labor and Statistics listed nonfatal occupational injuries involving days away from work caused by open wounds or burns at an incidence rate of 30.8 per 10,000 workers. In the U.S. alone, >6 million individuals develop chronic skin ulcers annually (1). The restoration or augmentation of cutaneous wound healing has long been an elusive goal for health care professionals.

Skin wound repair in most mammals follows a similar orderly sequence of events that are well known and have been thoroughly reviewed elsewhere (2). The inflammatory response, which precedes wound healing, is thought to be primarily involved in stemming infection and removal of cellular debris. However, indirect evidence suggests that inflammatory cytokines may be involved in modulating the healing process. For instance, glucocorticoids decrease the expression of proinflammatory cytokines in wounds and interfere with wound repair (3). Furthermore, elimination of macrophages, a major source of cytokines, from a wounded site delays wound healing (4). A study by Fahey et al. (5) demonstrated an association in streptozotocin-induced diabetic mice between lower levels of IL-6 in wound fluids and impaired wound healing. Impaired wound healing is a common complication among individuals with diabetes mellitus (6). IL-6 expression in dermal fibroblasts also decreases with age, which may contribute to delayed cutaneous wound healing in the elderly (7).

IL-6 is a pleiotropic cytokine that is involved in the growth and differentiation of numerous cell types (8). It is readily detected in cutaneous wounds (3, 9) and in the skin is produced primarily by epidermal keratinocytes, whereas macrophages, Langerhans' cells, and fibroblasts in the dermis represent minor sources of the cytokine (10). IL-6 is mitogenic for keratinocytes (11–13) and increased levels have been associated with a number of skin pathologies, such as psoriasis (12), scleroderma (14), and systemic lupus erythematosus (15). Overexpression of IL-6 in the skin of normal rats induces epidermal proliferation and inflammation (13), whereas transgenic mice overexpressing IL-6 display little more than a thickened stratum corneum (16). Although it seems that

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the role of IL-6 is well established in disease conditions, little is known about its role in skin repair.

It is well known that cutaneous healing in mice and rats is very similar to humans, and mice are a well-characterized model of human wound healing (5, 9, 17). In this study, we describe the role of IL-6 in the mouse using a punch biopsy model of skin wound healing. The punch biopsy method offers a convenient model to assess wound closure, re-epithelialization, and granulation tissue formation. We also investigate the use of recombinant protein and gene therapy as therapeutic interventions in delayed wound healing.

MATERIALS AND METHODS

In vivo wounding

Experimental animals 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. Male wild-type (C57BL/6J) mice (Jackson, Bar Harbor, Me.) or IL-6–deficient (C57BL/6J-*IL-6*^{tmKopf}) mice (graciously provided by Dr. H. Bluethmann, F. Hoffmann-La Roche, Switzerland) (18) weighing 22–28 g and ~8 to 12 wk old were housed in polycarbonate cages containing hardwood chip bedding at room temperature (21±2°C) on a 12-h light-dark cycle. Mice were anesthetized by i.p. injection with 80 mg/kg pentobarbital, and the left flank was clipped and swabbed with Betadine (Purdue Frederick, Norwalk, Conn.) and 70% ethanol three times before wounding. Four-millimeter punch biopsies were performed on the shaved area. Glucocorticoid-treated wild-type mice were dosed with 1 mg/kg/day s.c. for 7 days before, and each subsequent day after, wounding. After various healing periods, wounds were photographed or wound tissue was collected (2–3 mm border was excised around the wound) and preserved by flash-freezing for other studies or in buffered formalin for histology. Histological sections were stained with Hematoxylin and Eosin unless otherwise noted.

Determination of IL-6 mRNA expression by *in situ* hybridization

In situ hybridization was performed as a modification of the method of Howie (19). Briefly, paraffin-embedded formalin-fixed tissues were dewaxed in xylene, rehydrated in ascending alcohols, and digested for 1 h at 37°C in proteinase K (1 μg/ml). The slides were fixed in 0.4% paraformaldehyde in PBS for 20 min at 4°C. Twenty nanograms of biotinylated mIL-6 probe (R&D, Minneapolis, Minn.) was added to 50 μl of hybridization buffer (0.6M NaCl, 0.1% sodium pyrophosphate, 0.2% polyvinylpyrrolidine, 0.2% Ficoll, 5 mM EDTA, 50 mM Tris-HCl, 10% dextran sulfate, 10% sheared salmon sperm DNA, 50% formamide, pH 7.6). The slides were then incubated at 37°C in a humid atmosphere for 16 h with the probe solution. After incubation, the slides were washed under decreasingly stringent conditions in sodium citrate buffer. Slides were washed in tris buffered saline for 15 min and twice in buffer #1 (100 mM Tris HCl, 150 mM NaCl, pH7.4) for 5 min. The slides were blocked in buffer #1 containing 3% normal goat serum, (#S-1000, Vector, Burlingame, Calif) for 30 min. The slides were incubated for 5 h at room temperature in a humid chamber with a 1:2000 dilution of nutri-avidine alkaline phosphatase conjugate (Pierce, Rockford, Ill.) in blocking buffer. The slides were washed twice for 10 min and developed overnight in NBT/BCIP solution (Sigma, St. Louis, Mo.) containing 0.5% levamisole (Vector). After development, the slides were rinsed in dH_2O, dried and coverslips mounted.

In vivo AP-1 expression

Male mice carrying the AP1-luciferase transgene (graciously provided by Drs. M. Ding and V. Vallyathan, CDC/NIOSH, Morgantown, W.V.), originally developed by Rincon and Flavell (20), were crossed with DBA2 (SASCO, Omaha, Nebr.) female mice as described previously (21). The F1 offspring were screened by quantifying both basal- and TPA-induced levels of luciferase activity as an indicator for the presence of the AP-1-luciferase reporter gene. AP-1 transgenic mice were wounded as described. Tissue from wounded and nonwounded sites were taken from mice 16 h after wounding and placed in lysis buffer (200 μl/10 mg tissue) overnight at 4°C. The luciferase activity of the tissue supernatant obtained after lysis was measured by a luminometer.

EMSA

Transcription factor DNA binding activity was determined by a modification of the gel electrophoresis DNA binding assay described by Schreiber et al. (22). Briefly, AP-1 and Stat 3 oligonucleotide (Santa Cruz Biotech, Santa Cruz, Calif.) was labeled with γ^{32}P-ATP (NEN/Dupont, Boston, Mass.) using 6–10 U of T4 polynucleotide kinase (USB/Amersham, Cleveland, Ohio). The reaction mixture contained 40 μg of nuclear protein in 20 mM Hepes, pH 7.9, 4% Ficoll 400, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.25 mg/ml bovine serum albumin, 0.1 mg/ml of sheared salmon sperm DNA, and ~0.1 ng (2×10^5 cpm) of specific probe. Protein-DNA complexes were separated on a 4% nondenaturing polyacrylamide gel. The gels were electrophoresed at 125V in 50 mM Tris borate-EDTA buffer, dried, and exposed to a phosphorimager screen. For characterization of DNA-binding activity, the nuclear protein extracts were preincubated for 10 min before the addition of labeled probe with a 100-fold excess of unlabeled wild-type or mutant oligonucleotide. Nuclear protein from the liver of a C57BL/6 mouse exposed to 0.1 ml/kg of carbon tetrachloride was used as a positive control. This procedure has been shown to induce abundant AP-1 translocation in the liver (23).

Murine IL-6 expression plasmid

The full-length murine IL-6 cDNA from a wild-type mouse liver total RNA was amplified by PCR using the primers, 5′-gggaagcttcgctatgaagttcctctctgca and 5′-ggggaattccactaggtttgccgagtaga, which contain restriction sites for *Eco*RI and *Hin*dIII. The resulting PCR product was digested with *Eco*RI and gel purified (Qiagen, Valencia, Calif.). The IL-6 fragment was ligated into the expression plasmid pCMV (Stratagene, LaJolla, Calif.). XL1-blue supercompetent cells (Stratagene) were transformed with the resulting ligation reaction and were cultured on LB agar plates containing 25 μg/ml kanamycin. Colonies from the plate were grown in LB broth containing 25 μg/ml kanamycin, and plasmid was purified from each culture. Each plasmid was assessed for the presence of the IL-6 insert via PCR, restriction enzyme digestion, and dideoxy sequence analysis. Plasmids containing the proper murine IL-6 gene were assessed for activity by injecting 40 μg of the plasmid intradermally into IL-6 KO mice.

One plasmid (pCMVIL-6.2) was identified to have a sufficient level of expression in mouse skin.

RESULTS

IL-6 KO mice display delayed wound healing

IL-6 deficient (IL-6 KO) and wild-type control mice were subjected to full-thickness 4-mm punch biopsies on the left dorsal shaved flank and monitored for up to 15 days. Impairment of wound closure was noted in IL-6 KO mice 5 days after wounding based on gross morphology (**Fig. 1*a*** vs. **1*b***), histology (Figs. 1*c–f*), and wound area (mm^2) (**Fig. 2*a***). IL-6 KO mice displayed significantly decreased inflammation, granulation tissue formation, microvascularization, and incomplete re-epithelialization (Fig. 1*e, f*). This impairment was noted for as long as 15 days, nearly triple the normal healing time (Fig. 2*a*).

Localization of IL-6 mRNA synthesis in wound tissue

In situ hybridization was performed on sections of skin wound tissue from wild-type mice to localize IL-6 mRNA expression in cutaneous wounds. Figure 2*b* shows that IL-6 is expressed in epithelial keratinocytes at the wound edges and in macrophages and fibroblasts in the dermis (Fig. 2*d*). Epithelial IL-6 expression was not apparent distally from the wound, nor was there appreciable IL-6 expression in unwounded skin sections (not shown). IL-6 KO mice did not express IL-6 in either the dermis or epidermis of cutaneous wounds (not shown).

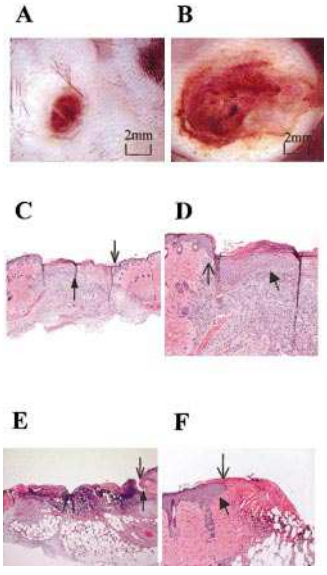

Figure 1. Wound healing in IL-6 KO mice. Representative photographs of 5-day-old wounds from wild-type (*A*) and IL-6 KO mice (*B*). Skin wound area from a 5-day-old wound in a wild-type mouse, 5× magnification (*C*) and 10× magnification (*D*). Closed arrow indicates epithelial layer, open arrow indicates wound edge. Skin section from 5-day-old wound in IL-6 KO mouse displaying lack of re-epithelialization, decreased granulation tissue, and decreased inflammation, 5× magnification (*E*) and 10× magnification (*F*). Closed arrow indicates epithelial layer, open arrow indicates wound edge.

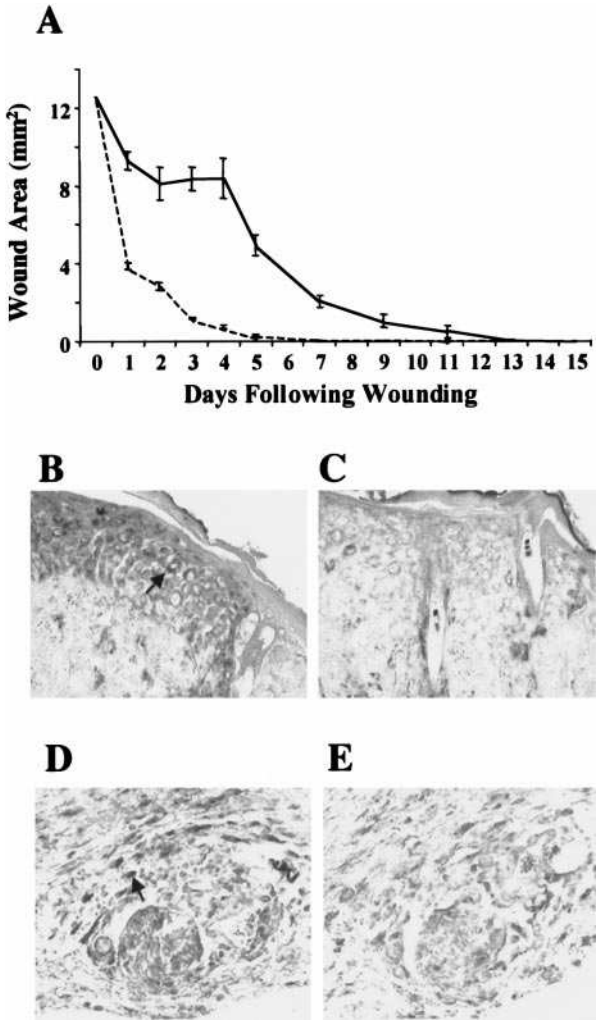

Figure 2. Time course of wound healing and IL-6 expression in wounds. Photographs of wounds were digitized and analyzed for wound area using NIH image 1.7 (*A*). Wound area was determined by calculating wound size relative to a 4 mm diameter circular paper cutout placed next to the wound in each photograph. Experimental groups consisted of 10 animals per group, and data are expressed as means ± SE of the wound area. IL-6 mRNA expression during wound healing (*B–F*). Fifteen-micrometer sections of wound tissue from wild-type mouse were taken 24 h after wounding and hybridized with biotinylated mouse IL-6 cDNA probe. IL-6 mRNA expression in leading-edge keratinocytes (*B*) and adjacent section not exposed to probe (*C*). Histological section of wound dermis from wild-type mouse 24 h after wounding, hybridized with biotinylated mouse IL-6 cDNA probe showing expression of IL-6 in macrophages and fibroblasts of the dermis (*D*) and adjacent section not exposed to probe (*E*).

Transcriptional activation associated with IL-6

The activation of the transcription factors AP-1, CEBP/β, and STAT 3 is associated with IL-6 activity in various tissues. To determine if AP-1 was activated in skin by wounding, transgenic mice carrying a AP1-luciferase transgene were wounded and luciferase activity was assessed. On average, luciferase activity was 1,500-fold higher in wounded skin

than in intact skin (**Fig. 3*a***). To further characterize transcriptional events after wounding, electrophoretic mobility shift assays (EMSA) were performed on cutaneous wound tissue from wild-type and IL-6 KO mice. Significant AP-1 activation occurred in wild-type mice 16 h after wounding, which was not manifested in IL-6 KO mice (Fig. 3*b*). However, activation of Stat 3 was not apparent in wounded or nonwounded skin from either wild-type or IL-6 KO mice (Fig. 3*c*). C/EBP (NF-IL-6) activation was also examined but not found to differ between wild-type and IL-6 KO mice at the time points examined (not shown).

The effect of rmIL-6 or IL-6 gene therapy on wound healing in IL-6 KO mice

IL-6 KO mice were administered 1 mg/kg s.c. of rmIL-6 1 h before wounding. This dose was shown previously to induce liver regeneration in IL-6 KO mice after partial hepatectomy (24). Treatment with rmIL-6 allowed healing to occur at a similar rate to that in wild-type mice. Histological examination revealed that wounds in rmIL-6–treated IL-6 KO mice re-epithelialized normally and had well-formed granulation tissue (**Fig. 4*a*, *b***). Reversal of delayed wound healing in IL-6 KO mice was also evaluated after injection of a mammalian expression plasmid construct containing the full-length murine IL-6 cDNA linked to a CMV promoter (pCMVIL-6.2). IL-6 mRNA was expressed at levels detectable by RT-PCR when 40–50 μg of plasmid was injected intradermally into IL-6 KO mice (not shown). When injected with 50 μg of pCMVIL-6.2 16 h before wounding, 60% of the mice displayed wound closure 5 days later. Re-epithelialization and granulation tissue formation in these mice was similar to wild-type or IL-6 KO mice treated with rmIL-6 (Fig. 4*c*). RT-PCR confirmed IL-6 expression only in those mice that healed (4 out of 6) and lack of expression in those that displayed impaired healing (Fig. 4*e*). IL-6 expression was no longer apparent in any of the plasmid-treated mice 5 days after wounding, indicating the expected transient nature of gene transfer in keratinocytes (not shown).

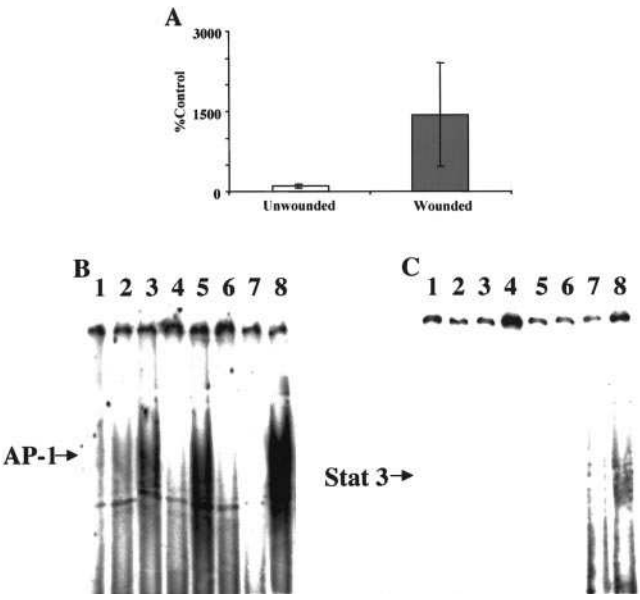

Figure 3. AP-1 and Stat 3 transcriptional activation in wound tissue. *A*) Luciferase activity in wounded or unwounded skin of AP1-luciferase transgenic mice. Data were normalized to tissue weight and expressed as a percent of control (n=6, ±SE). *B*) EMSA of AP-1 activation in wound tissue from wild-type and IL-6 KO mice. Lane 1, wild-type not wounded; lane 2, IL-6 KO not wounded; lanes 3 and 5, wild-type 15 h after wounding; lanes 4 and 6, IL-6 KO 15 h after wounding; lane 5, replicate experiment as in lane 3; lane 6, replicate experiment as in lane 4; lane 7, CCl_4-treated mouse liver positive control + 1,000-fold excess of nonlabeled AP-1 probe; lane 8, CCl_4-treated mouse liver positive control. *C*) Stat 3 activation: lane 1, wild-type not wounded; lane 2, IL-6 KO not wounded; lane 3, wild-type 15 h after wounding; lane 4, IL-6 KO 15 h after wounding; lane 7, CCl_4-treated mouse liver positive control containing 1,000-fold excess of nonlabeled AP-1 probe; lane 8, mouse liver positive control.

The effect of rmIL-6 on wound healing in glucocorticoid-treated wild-type mice

To determine if IL-6 alone could be of use to aid in wound healing, mice were immunosuppressed by dosing with 1 mg/kg/day of dexamethasone for 7 days before wounding and each day thereafter. This dosing regimen has been shown to inhibit cytokine and growth factor expression after cutaneous wounding (25). As shown in **Fig. 5**, a single dose of 1 mg/kg of rmIL-6 1h before wounding significantly reduced wound area and healing time in mice treated with dexamethasone.

DISCUSSION

Although it seems that the role of IL-6 is well established in immune and inflammatory diseases, little is known about the role of the cytokine in normal physiological processes, such as cutaneous wound healing. IL-6 is produced in the wound by epidermal keratinocytes, dermal fibroblasts, and macrophages (Fig. 2) (26), and it affects multiple processes that are related to wound healing. For example, IL-6 is proinflammatory and, after wounding, increases adhesion of neutrophils to dermal fibroblasts (27). Several studies have reported that IL-6 is a mitogen for keratinocytes *in vitro* (11, 12) and *in vivo* (13). IL-6 may also assist wound healing indirectly by modulation of growth factors or their receptors. In this respect, IL-6 induces KGF expression in fibroblasts (28, 29) and EGF receptor in keratinocytes *in vitro* (30). When overexpressed in

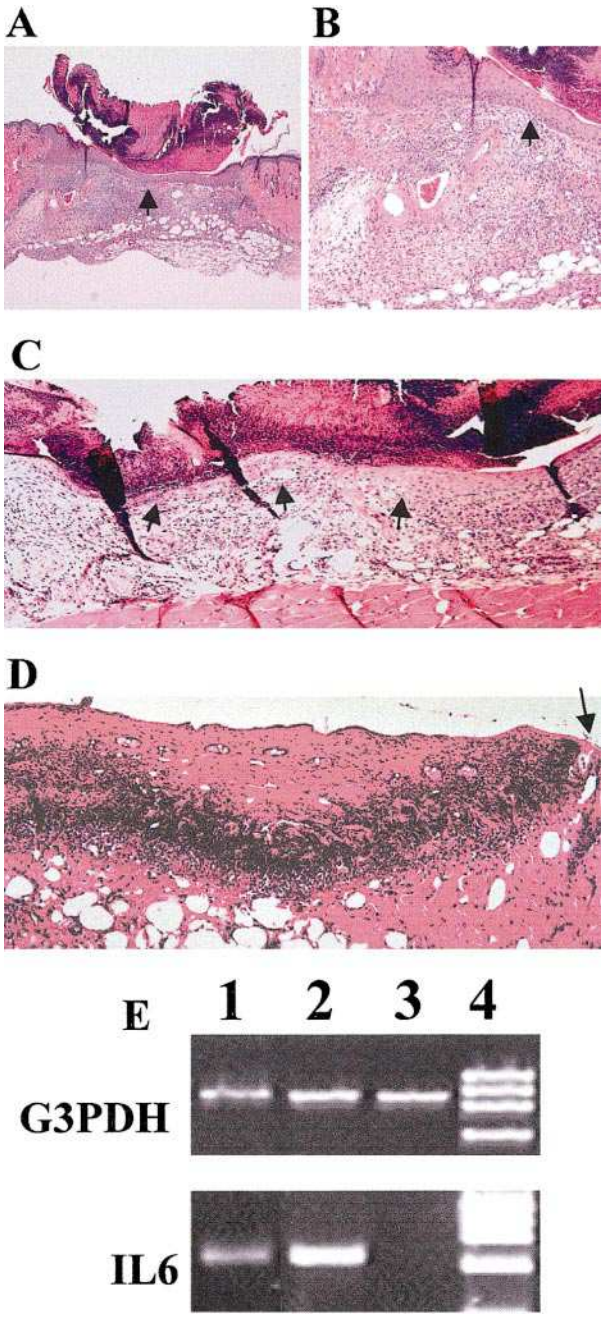

Figure 4. rmIL-6 administration or IL-6 gene transfer reverses healing deficiency in IL-6 KO mice. 5× (*A*) and 10× (*B*) microscopy of 5-day-old wound section from an IL-6 KO mouse treated with 1 mg/kg rmIL-6 displaying normal re-epithelialization and well-formed granulation tissue similar to control. Arrow indicates epithelial layer. *C*) Histology of 5-day-old wound from mouse treated with pCMVIL-6.2 expressing IL-6. Arrow indicates complete epithelial layer. *D*) Histology of 5-day-old wound from a mouse treated with pCMVIL-6.2 but not expressing IL-6, displaying lack of epithelial bridge and minimal granulation tissue. Arrow indicates leading edge of epithelial layer. *E*) IL-6 expression by RT-PCR from wound tissue of pCMVIL-6.2 treated IL-6 KO mice 15 h after intradermal injection of 50 μg plasmid. Total RNA was isolated from 4-mm skin punch biopsy, and expression was examined by RT-PCR for mIL-6 as described in Materials and Methods. Lane 1, positive control; lane 2, corresponding skin from IL-6 KO mouse (see panel *C*) expressing IL-6; lane 3, corresponding skin from IL-6 KO mouse treated with pCMVIL-6.2 not expressing appreciable levels of IL-6 (see panel *D*); lane 4, molecular weight marker.

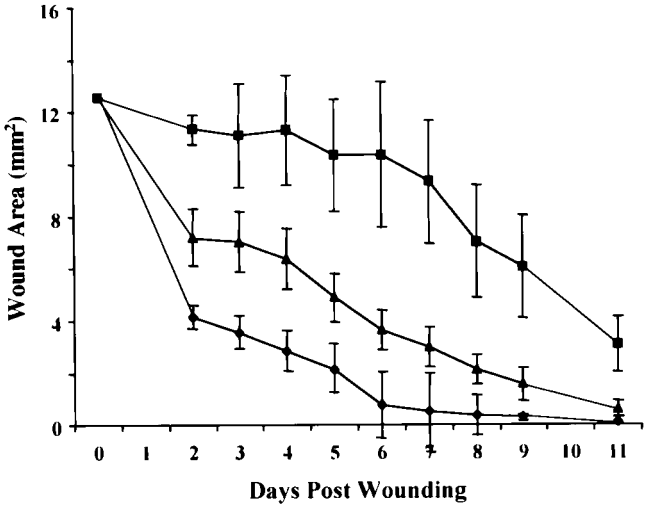

Figure 5. rmIL-6 administration promotes wound healing in dexamethasone-treated mice. Mice were dosed with 1 mg/kg/day dexamethasone for 7 days before and 11 days after wounding. Mice were wounded as described, and wounds were photographed at various time points. Photographs of wounds were digitized and analyzed for wound area using NIH image 1.7. Experimental groups consisted of 10 animals per group, and data are expressed as means ± SE of the wound area. ♦ = vehicle treated, ■ = dexamethasone treated, ▲ = dexamethasone + rmIL-6 treated.

normal rat skin, IL-6 induces TGFα (13), a potent keratinocyte mitogen, and TGFα in turn induces IL-6 in keratinocyte cultures, indicating a paracrine interaction (31). Herein, we demonstrate that IL-6 expression is intimately involved in cutaneous wound healing, as IL-6KO mice display impaired wound healing manifested as deficiencies in re-epithelialization, granulation tissue formation, and inflammation. Preliminary data suggest that the mechanism by which IL-6 controls wound healing is indirect, involving the regulation of genes involved in growth factor expression.

IL-6 has been associated with the activation of the transcription factor AP-1 in liver (24), cultured fibroblasts (32), and various cell lines (33). AP-1 translocation has also been linked to keratinocyte proliferation (34, 35). We observed that wounding is associated with both AP-1 translocation and IL-6 expression, and their peak activity coincides with similar kinetics ~16 h after wounding (Fig. 3*a,b*). However, there is another peak of AP-1 induction that occurs 2 h after wounding that is not associated with IL-6 expression. It is possible that other inflammatory mediators, possibly proinflammatory cytokines such as TNF-α and IL-1, might modulate early transcriptional activation during wound healing. TNF-α and IL-1β are transiently induced immedi-

ately after cutaneous injury, peaking at ~3 h, after which both cytokines decrease to near basal levels by 10 h (9). Both of these proinflammatory cytokines induce AP-1 translocation in various tissues (36) and are readily induced in the skin of normal and IL-6 KO mice after wounding (Gallucci et al., unpublished observations). In contrast, IL-6 is induced more slowly after wounding, reaching maximal induction after 12 h before gradually returning to basal levels after 24 h (9), thus coinciding with AP-1.

The activation of Stat 3 (37, 38) and C/EBPβ, similar to AP-1, is associated with IL-6 stimulation in various tissues. However, we observed that activation of Stat 3 and C/EBP does not differ between wild-type or IL-6 KO mice during the early stages of cutaneous wound healing (Fig. 3*c*). This is consistent with earlier studies suggesting that both Stat 3 (39) and C/EBPβ (40–43) are associated with differentiation, rather than the proliferation of keratinocytes. Thus, these transcription factors may be associated with the end stages of wound healing and is activated only after re-epithelialization.

Transgenic animals can show variations in responses that are secondary to the gene that was altered. To determine that the deficiencies noted in wound healing were due in fact, to the lack of bioactive IL-6, transgenic animals were reconstituted with rmIL-6 or a mammalian expression plasmid containing consensus cDNA sequence for murine IL-6. IL-6 KO mice treated with the recombinant cytokine (Fig. 4*a*) or IL-6 plasmid (Fig. 4*c*) healed similarly to control mice, indicating that IL-6 has a direct influence on wound healing that is not associated with nonspecific phenotypic changes in IL-6 KO mice. This is consistent with studies showing that IL-6 KO mice express normal levels of functional IL-6 receptors (24, 44).

It is well known that impaired cutaneous healing occurs in individuals receiving glucocorticoids or other immunosuppressive therapies (45). Although glucocorticoid treatment suppresses a host of cytokines and growth factors, the role IL-6 plays in cutaneous healing of immunosuppressed mice is not known. Herein we show that rmIL-6 can significantly augment wound healing in dexamethasone-treated mice (Fig. 5), indicating that IL-6 is a pivotal signal during wound healing and a potential therapeutic for side effects associated with immunosuppressive therapy. However, the wound-healing response was not entirely restored by IL-6 treatment alone. Given the broad inhibitory activity of glucocorticoids on cytokine expression, it is not unlikely that other cytokines might cooperate with IL-6 in eliciting a complete healing response.

Although possible side effects from IL-6 protein could make systemic administration troublesome, local and transient expression of IL-6, such as that produced in this model of gene therapy, would be more promising because expression is transient and confined to the site of administration, minimizing undesirable side effects. Intradermal delivery of expression plasmid is not the most efficient method of gene transfer into skin (46), as evidenced by the variations we observed in IL-6 gene expression in our model (66% success rate). However, the efficacy of gene transfer could be improved by the use of other methods such as an adenovirus based viral vector, or 'gene-gun', which delivers microscopic particles coated with plasmid directly into the tissue. Experiments designed to assess the effectiveness of these treatments on immunosuppressed and diabetic animal models are currently under investigation in this laboratory. FJ

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