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Research Article

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One Systemic Administration of Transforming Growth Factor-β1 Reverses Age- or Glucocorticoid-impaired Wound Healing

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Abstract

The role of intravenously administered recombinant human transforming growth factor-β1 (rhTGF-β1) on the healing of incisional wounds in rats with impaired healing due to age or glucocorticoid administration was investigated. The administration of methylprednisolone to young adult rats decreased wound breaking strength to 50% of normal control. Breaking strength of incisional wounds from 19-mo-old rats was decreased ~ 27% compared with wounds from normal healing young adult rats. A single intravenous administration of rhTGF-β1 (100 or 500 μg/kg) increased wound breaking strength from old rats or young adult rats with glucocorticoid-induced impaired healing to levels similar to normal healing control animals when determined 7 d after injury. Even though the circulating half-life of systemically administered rhTGF-β1 is < 5 min, a sustained stimulatory effect on extracellular matrix secretion was evident in glucocorticoid-impaired rats when rhTGF-β1 was administered at the time of wounding, 4 h after wounding, or even 24 h before wounding. These observations indicate a previously unrecognized potential for the active form of TGF-β1 to profoundly influence the wound healing cascade after brief systemic exposure. (*J. Clin. Invest.* 1993. 92:2841–2849.) Key words: transforming growth factor-β1 • breaking strength • impaired healing

Wound healing proceeds through a series of coordinated cellular- and cytokine-mediated events that culminates in the restoration of functional integrity of tissue. Impaired wound healing may be a consequence of normal aging, metabolic derangements, or therapeutic intervention. Elderly patients heal more slowly than young patients, which is evident clinically as increased rates of wound dehiscence, prolonged recovery time, and increased duration of hospital care (see review by Jones and Millman [1]). Studies with old rodents correlate with the clinical observations in humans. The rates of cellular proliferation and revascularization, and the deposition and remodeling of collagen at wound sites, are decreased in old compared with young rodents, changes that are associated with slower healing and reduced wound strength (2–4). Similar observations concerning impaired wound healing have been made in glucocorticoid-treated animals (5, 6).

The local production of growth factors such as TGF-β1 or PDGF has been associated with various stages of tissue repair (7, 8). With the recent availability of large amounts of these factors through recombinant techniques, several investigators have shown that the local application of growth factors accelerates tissue repair in animal models of wound healing (9–11). TGF-β1, a product of a variety of cell types, including platelets, monocyte/macrophages, and fibroblasts, is produced in a latent form that must undergo activation, possibly through local upregulation of urokinase type plasminogen activator, to yield a 25-kD homodimeric protein (12, 13). The single topical application of active TGF-β1 to wounds accelerates the process of repair (14–17). We previously showed that the repeated application of the active form of recombinant human (rh)[1] TGF-β1 to a wound was of greater benefit than a single application if healing was delayed sufficiently to adequately evaluate the extended repair process (18). One observation that arose from these studies was a marked increase in healing after a second application of rhTGF-β1 to the wound site. Also noted during the evaluation of systemic effects of rhTGF-β1 was an exaggerated fibroproliferative healing response at a distant locus, the site of intramuscular anesthetic injection of ketamine (our unpublished data). We interpreted these results as possibly indicating that exposure to TGF-β1 altered the subsequent response of cells to additional growth factors. We therefore sought to investigate this possibility in a more defined manner by administering TGF-β1 systemically in order to determine the influence of a single exposure on the healing of wounds in old rats and glucocorticoid-impaired young adult rats.

We report here that the single systemic administration of rhTGF-β1 to young adult rats in which healing was impaired by glucocorticoids or to old rats increased the breaking strength of incisional wounds to levels similar to that of normal young adult rats. In addition, prevention of glucocorticoid-induced impaired healing could be accomplished with the single systemic administration of TGF-β1 as early as 24 h before wounding. Although it had been reported previously that topically applied rhTGF-β1 accelerated the healing of incisional wounds in normal young adult rats or in rats whose healing response had been impaired by the administration of glucocorticoids (18, 19) or antimetabolites (20), it was unclear whether age-related changes that underlie the delayed healing in older animals would respond to rhTGF-β1. Therefore, additional studies were done to evaluate the effects of topically applied rhTGF-β1 (1–4 μg/wound in 50 ml of 3% methylcellulose) in old rats.

A portion of this work has been published in abstract form (1991. *J. Cell. Biochem.*) 15S:191).

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1. *Abbreviation used in this paper:* rh, recombinant human.

Methods

Source and preparation of TGF-β1. rhTGF-β1 was cloned (21) and expressed in Chinese hamster ovary (CHO) cells, and was aseptically prepared in 20 mM sodium acetate buffer at pH 5.0. The placebo control was formulated in phosphate buffer without rhTGF-β1. Topical formulations with or without rhTGF-β1 were prepared in a similar manner and contained 3% methylcellulose as the placebo. All materials were stored at 5°C until use.

Incisional wounds. All animal studies were performed in accordance with guidelines from the National Institutes of Health and the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Rats were anesthetized with a ketamine/xylazine mixture administered intramuscularly. Four full-thickness transverse incisions were made at sites on the back and the edges were opposed with two equally spaced interrupted 4-0 stainless steel sutures as previously described (18). All rats were killed with an overdose of CO_2 7 d after wounding. Two samples from each wound were removed from the incision site to the level of the panniculus carnosus, uniformly trimmed in width and length to assure exposure of the ends of the incision, and fixed in 10% neutral buffered formalin for 7 d. Formalin fixation was done for facilitation of handling of the fragile wound tissue. Although fixation increases collagen crosslinking and absolute breaking strength of wounds, the increase parallels that seen without formalin fixation, thus permitting intergroup comparisons (22, 23). Breaking strength was performed in a blinded manner on coded samples using a calibrated tensometer (Instron Universal Testing Instrument 1011; Instron Corp., Canton, MA) as previously described (18). Additional sections were processed by routine methods for histological examination.

Aged rat studies. 19-mo-old male Fischer rats, (395–465 g; Harlan Sprague Dawley, Inc., Hayward, CA) were administered a single intravenous dose of PBS as the placebo or rhTGF-β1 (100 or 500 μg/kg) 5 min before wounding. Parallel control studies were done with young adult (3 mo old) male Fischer rats (268–272 g) in order to compare the normal healing response of young adult to old rats. Wound strength and histological samples were assessed as described above.

Glucocorticoid-impaired rat studies. The healing response of adult male Sprague-Dawley rats (300–350 g; Charles River Breeding Laboratories, Wilmington, MA) was impaired by administration of methylprednisolone (5 mg/rat intramuscularly) at the time of wounding. The rats received rhTGF-β1 as a single intravenous dose (10, 100, or 500 μg/kg) or a PBS placebo at three different time points: 24 h or 5 min before, or 4 h after wounding. Wound strength and histological samples were assessed as described above.

Statistical analysis. Breaking strength measurements from the four wounds on each rat were averaged. Group means and SEM were calculated using the individual animal averages as raw data. The data were analyzed by ANOVA with comparisons made between rhTGF-β1-treated and control groups. When the overall F test indicated group differences, individual group means were compared using the Dunnett's t test (24).

Results

Topical rhTGF-β1 reverses age-related impairment of wound healing. 19-mo-old Fischer rats had a decrease in breaking strength of incisional wounds of ~ 27% when compared with 3-mo-old Fischer rats measured 7 d after wounding (Fig. 1).

The topical application of rhTGF-β1 (1 or 4 μg/wound) increased the breaking strength of incisional wounds in old rats to levels observed in normal healing young adult rats, a finding similar to that previously seen in glucocorticoid-impaired rats (18) (Fig. 1). Since wounds in aged rats could respond to rhTGF-β1, we examined the influence of systemic rhTGF-β1 in two rat models of impaired wound healing: aged and glucocorticoid impaired.

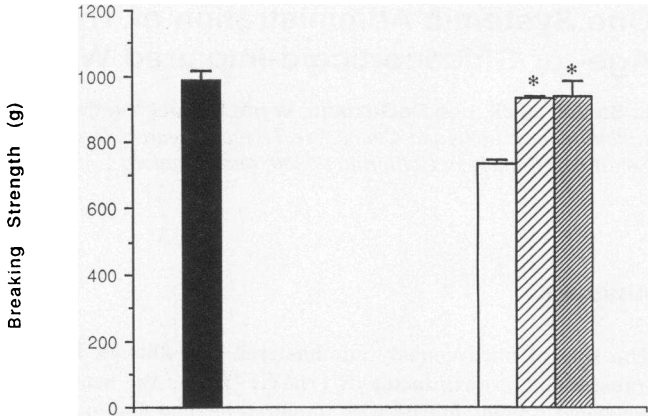


Figure 1. Topical rhTGF-β1 reverses age-related impairment of wound healing. The breaking strength of incisional wounds from old Fischer 344 rats receiving rhTGF-β1 topically in 3% methylcellulose or methylcellulose alone was compared. Young adult normal healing control rats (■; $n = 12$) received placebo. Aged-impaired healing rats ($n = 4$) received placebo (□) or rhTGF-β1 at 1 μg (▨; $n = 2$) or 4 μg (▨; $n = 2$) per wound as described in Methods. Data represent mean±SEM with significant differences at $^*P < 0.01$.

Systemic rhTGF-β1 reverses age-related impairment of wound healing. The breaking strength of incisional wounds from old rats administered a single intravenous dose of rhTGF-β1 (either 100 or 500 μg/kg) at the time of wounding was increased compared with wounds from rats treated with placebo (Fig. 2). rhTGF-β1 increased the breaking strength of the wounds to levels similar to that observed in normal healing young adult rats.

When histological sections from 7-d-old wounds were examined, wounds from the 19-mo-old rats administered rhTGF-β1 contained few inflammatory cells that appeared to be primarily macrophages as well as numerous fibroblasts. The extracellular matrix bridging the wounds appeared thick and was arrayed in an orderly mosaic pattern (Fig. 3 *A*). In con-

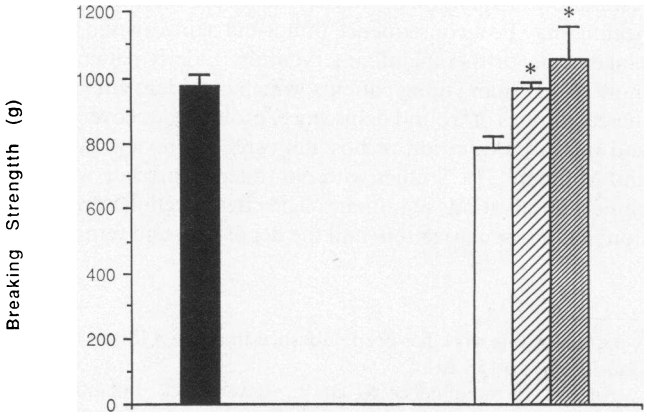


Figure 2. Intravenous rhTGF-β1 reverses age-related impairment of wound healing. The breaking strength of incisional wounds from old Fischer 344 rats administered PBS or rhTGF-β1 intravenously were compared. Young adult normal healing control rats (■; $n = 16$) were administered PBS intravenously before surgery. Aged rats with impaired healing were administered PBS (□; $n = 5$) or rhTGF-β1 at 100 μg/kg (▨; $n = 6$) or 500 μg/kg (▨; $n = 4$) intravenously before surgery. Data represent mean±SEM with significant differences at $^*P < 0.05$.

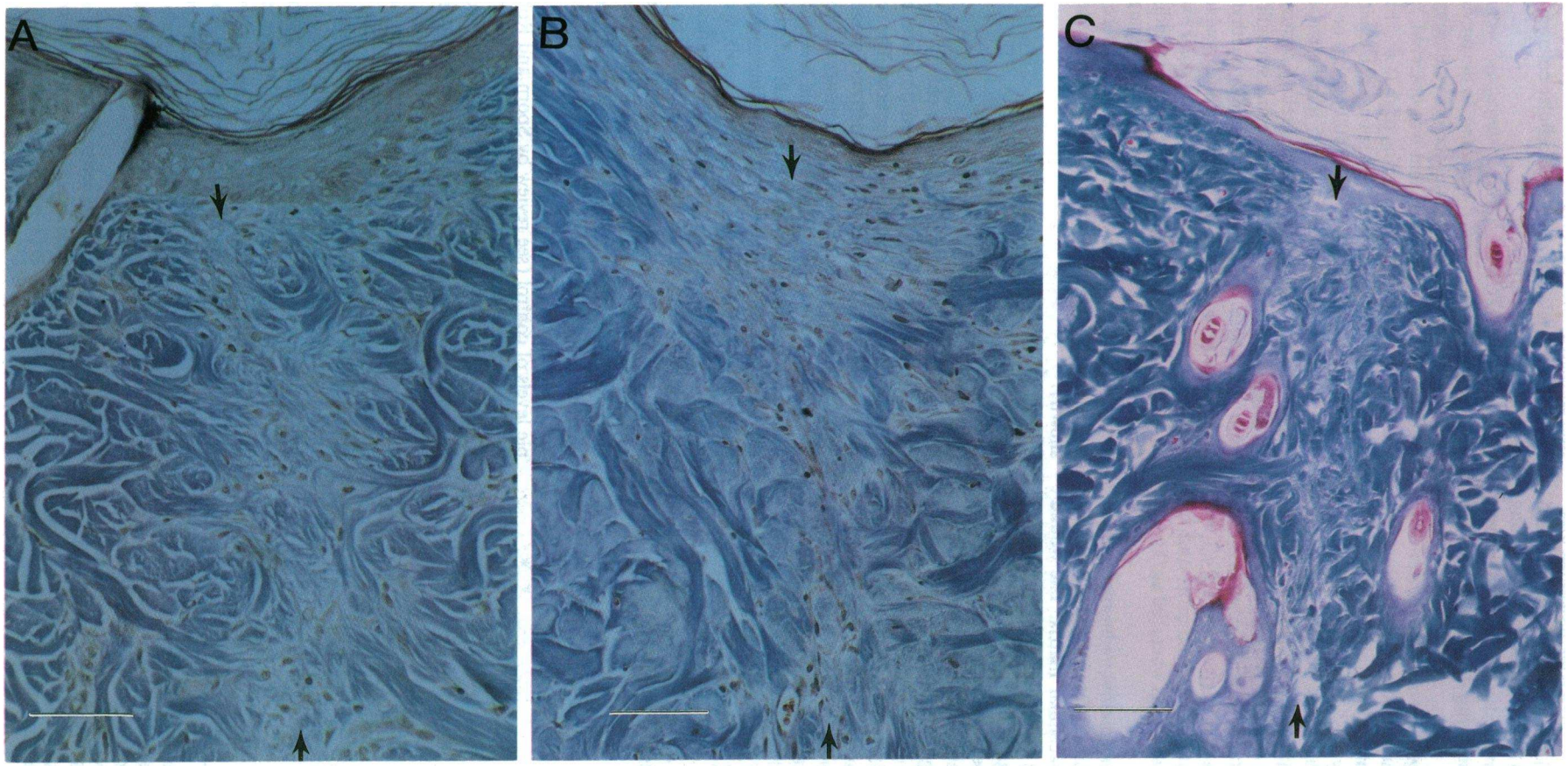


Figure 3. Photomicrographs of incisional wounds from old Fischer 344 rats administered PBS or rhTGF-β intravenously. (*A*) The incision site (*arrows*) of an old Fischer 344 rat administered rhTGF-β1 (100 μg/kg) at the time of wounding contains small mature collagen bundles transecting the plane of the incision (bar = 100 μ). (*B*) The incision site (*arrows*) of an old Fischer 344 rat receiving PBS transects the dermis with few vertically oriented collagen bundles parallel to the incision (bar = 100 μ). (*C*) The incision site removed from a young adult Fischer 344 administered PBS before surgery. The healing tissue contains vertically oriented collagen bundles as well as collagen bundles transecting the plane of incision (bar = 100 μ). Samples for histological examination were obtained from each wound. Cross sections of the wounds from the center of each scar were removed 7 d after injury and fixed in 10% neutral buffered formalin. Tissues were paraffin embedded and 4-μ sections stained with trichrome. Care was taken to maintain the same spatial orientation for mounting and sectioning all tissues. Photomicrographs are representative of the differences observed between groups.

trast, sites from PBS-treated rats contained an occasional macrophage with a loose extracellular matrix vertically oriented parallel to the incisions (Fig. 3 *B*). Incisional sites from young adult male Fischer 344 rats contained a mixture of collagen vertically oriented as well as transecting the plane of the incision (Fig. 3 *C*).

Systemic rhTGF-β1 reverses glucocorticoid-impaired wound healing. The administration of the glucocorticoid, methylprednisolone, to young adult rats (6 mo old) impaired the breaking strength of incisional wound by > 50% (Fig. 4). The single intravenous administration of rhTGF-β1 (100 or 500 μg/kg) to rats whose wound healing response was impaired by glucocorticoids increased the breaking strength of incisional wounds to that of control rats ($P < 0.01$) (Fig. 4). An increase in breaking strength of wounds from rats administered rhTGF-β1 was noted at 10 μg/kg (but not statistically significant) and increased to that of normal young adult rats when 100 μg/kg was given.

The wounds from rats receiving methylprednisolone, when examined 7 d after injury, contained few inflammatory cells and scant, loosely arranged extracellular matrix. The wound margins were clearly demarcated under polarizing light (Fig. 5 *A*). In contrast, wound margins from rats administered rhTGF-β1 (500 μg/kg) 5 min before surgery were difficult to detect, with wound margins that were barely distinguishable from the surrounding tissue (Fig. 5 *B*), similar to the normal healing rats (Fig. 5 *C*). The wounds from rhTGF-β1-treated rats were characterized by a moderate number of macrophages and fibroblasts and a densely arranged extracellular matrix. When examined by transmission electron microscopy, fibroblasts from the wound site of rats with impaired healing contained scant endoplasmic reticulum consistent with a low secretory state. Small quantities of amorphous extracellular matrix were present between cells (Fig. 6 *A*). Wounds from rats administered rhTGF-β1 (Fig. 6 *C*), however, were similar to normal control wounds (Fig. 6 *B*), containing numerous active fibroblasts with large amounts of rough endoplasmic reticulum consistent with enhanced synthetic activity. Large quantities of dense, well-organized extracellular collagen fibrils were evident in all preparations.

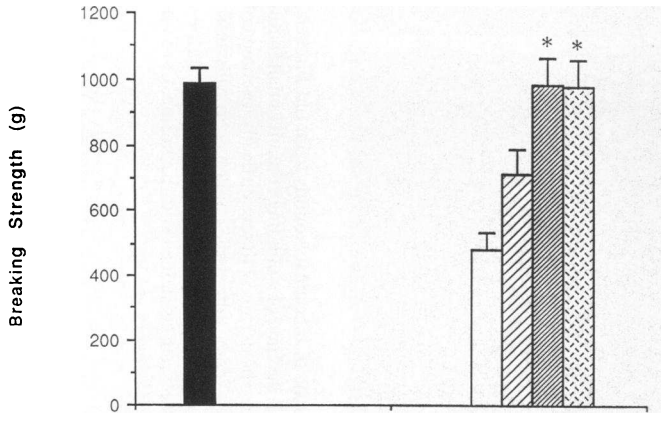


Figure 4. Intravenous rhTGF-β1 reverses glucocorticoid impairment of wound healing. Comparison of breaking strength of incisional wounds from rats administered PBS or rhTGF-β1. Normal healing rats (■) were administered PBS intravenously before surgery. Impaired healing rats were administered PBS (□) or rhTGF-β1 at 10 μg/kg (▨), 100 μg/kg (▨), or 500 μg/kg (▨) intravenously before surgery. Data represent mean±SEM from four to five rats per group with significant differences at $*P < 0.01$.

Systemic rhTGF-β1 reverses impaired wound healing whether given before or after wounding. In the previous studies, rhTGF-β1 was given immediately before wounding. In view of the profound repair response that was observed, we explored the relationship between the timing of rhTGF-β1 administration and the subsequent healing response. To examine this response further, rhTGF-β1 (100 or 500 μg/kg) or PBS was injected intravenously either 24 h or 5 min before, or 4 h after wounding. Wounds from normal healing control rats were again evaluated in parallel. The breaking strength of wounds from rats whose healing was impaired by methylprednisone and who received rhTGF-β1 were indistinguishable from normal healing control animals at each time point except at 500 μg/kg rhTGF-β1 administered 24 h before wounding (Fig. 7). When rhTGF-β1 was administered 48 h before surgery, results were marginal, and when administered 72 h before surgery, no reversal of steroid impaired healing was observed (data not shown).

Discussion

The decreased healing capacity of the elderly is the result of multiple factors, including reduced nutritional status, immunological competence, and local vascular flow. The cellular components required for healing of the elderly are present but onset is delayed temporally and healing progresses more slowly than in younger patients (3). Pharmacological levels of corticosteroids retard inflammation and wound healing by impairing the chemotaxis of inflammatory cells, inhibiting angiogenesis, and decreasing fibroblast proliferation and matrix synthesis (see review by Wahl [6]). TGF-β1, in contrast, stimulates neovascularization, macrophage chemotaxis, and proliferation of fibroblasts, as well as the synthesis and subsequent maturation of extracellular matrix. Although TGF-β1 is able to reverse many effects of corticosteroid administration and age-related phenomena, it is not clear how this reversal is mediated. Modulation of wound repair by TGF-β1 is complex and involves multiple levels of control (see review by Sporn and Roberts [25]), and depends on the presence of other growth factors or regulatory peptides (7, 26, 27), the target cell, and the cell's state of differentiation (for examples, see Janat and Liau [28] or Celada and Maki [29]). TGF-β1's action on neutrophils (30) and monocytes (31), as well as other cell types, is presumably mediated through membrane-bound receptors that when activated stimulate cytoplasmic and nuclear responses resulting in a cascade of cellular and extracellular events (32).

Because we used a single intravenous dose of rhTGF-β1 to modulate wound repair that was assessed 7 d later, it is impossible to pinpoint its course of action. However, we do know whether this effect was time dependent since the administration of rhTGF-β1 24 h before or 4 h after surgery prevented or reversed the glucocorticoid effect, and that administration of rhTGF-β1 72 h before wounding was without effect (data not shown).

Systemic clearance of the active form of TGF-β1 is rapid, with a circulating half-life of < 5 min with low doses (≤ 1 μg TGF-β1) (33), and < 11 min with higher doses (≤ 300 μg rhTGF-β1) (33a). Yet, the orderly cascade of events required

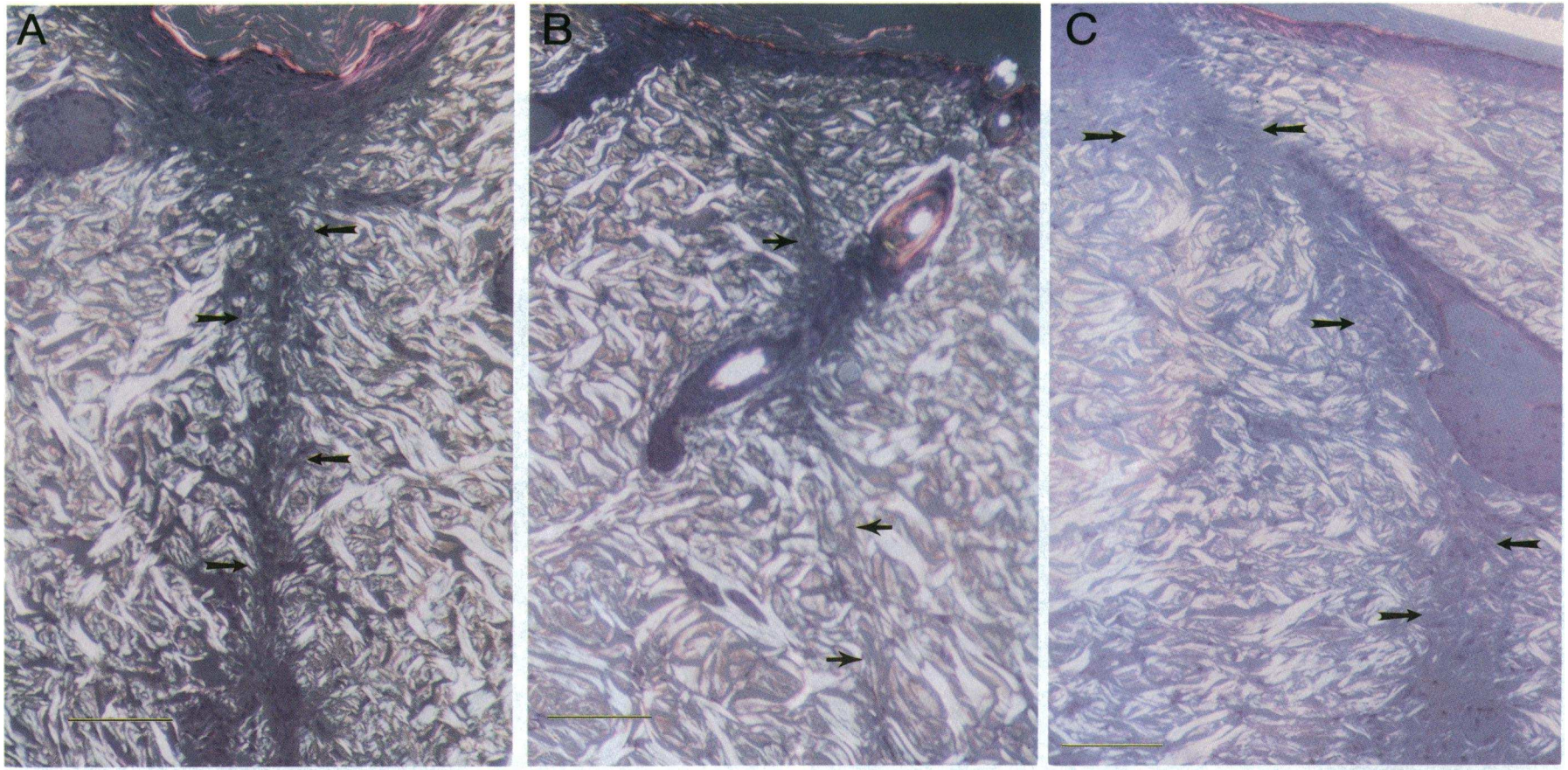
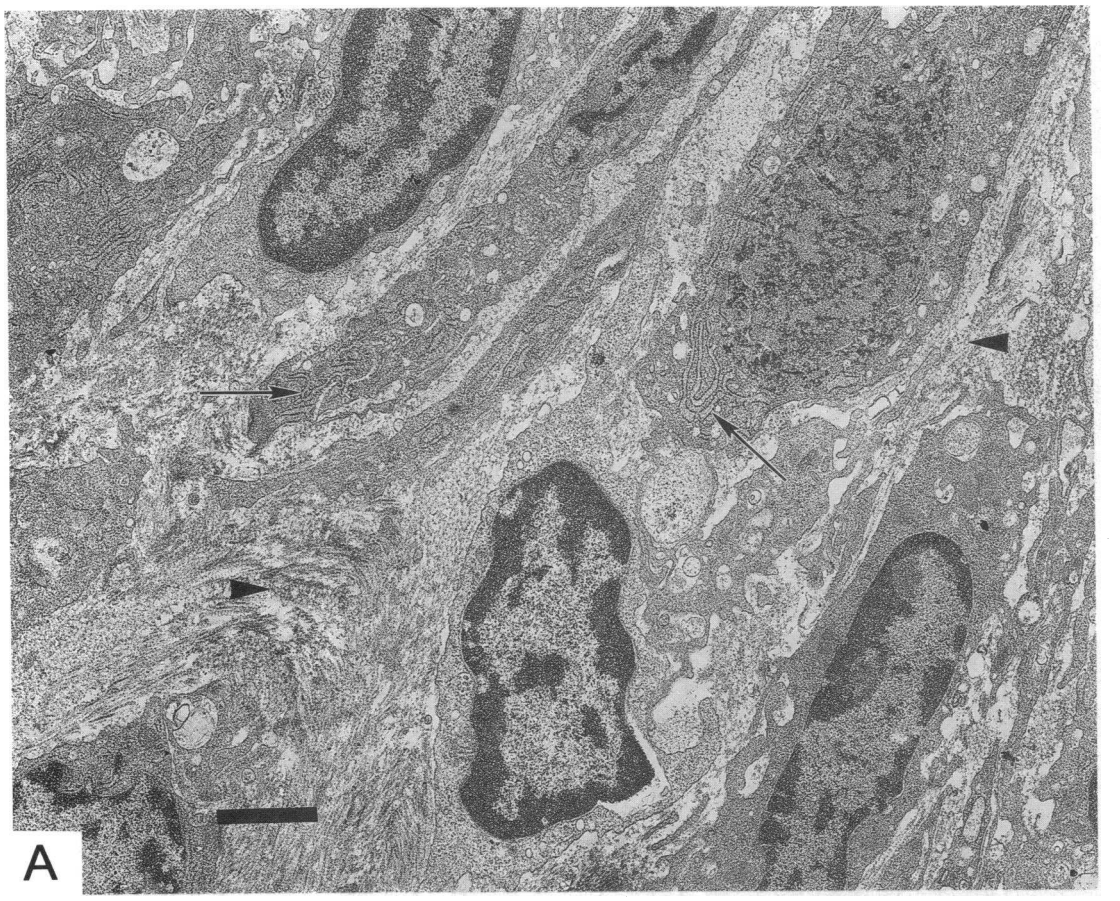


Figure 5. Photomicrographs of polarized histological sections of incisional wounds. (*A*) The incision site of an impaired healing rat treated with PBS alone is still obvious (*arrows*), transecting the dermis with few collagen bundles bridging the gap (bar = 100 μ). (*B*) The incision site of an impaired healing rat administered rhTGF-β1 (500 μg/kg) intravenously at the time of wounding is difficult to determine (*arrows*) and contains small mature collagen bundles transecting the plane of the incision (bar = 100 μ). (*C*) The incision site from a normal healing rat contains loose matrix crossing the defect (bar = 100 μ). Samples for histological examination were obtained from each wound. Cross sections of the wounds from the center of each scar were removed 7 d after injury and fixed in 10% neutral buffered formalin. Tissues were paraffin embedded and 4-μ sections stained with hematoxylin and eosin. Care was taken to maintain the same spatial orientation for mounting and sectioning all tissues. Photomicrographs are representative of the differences observed between groups.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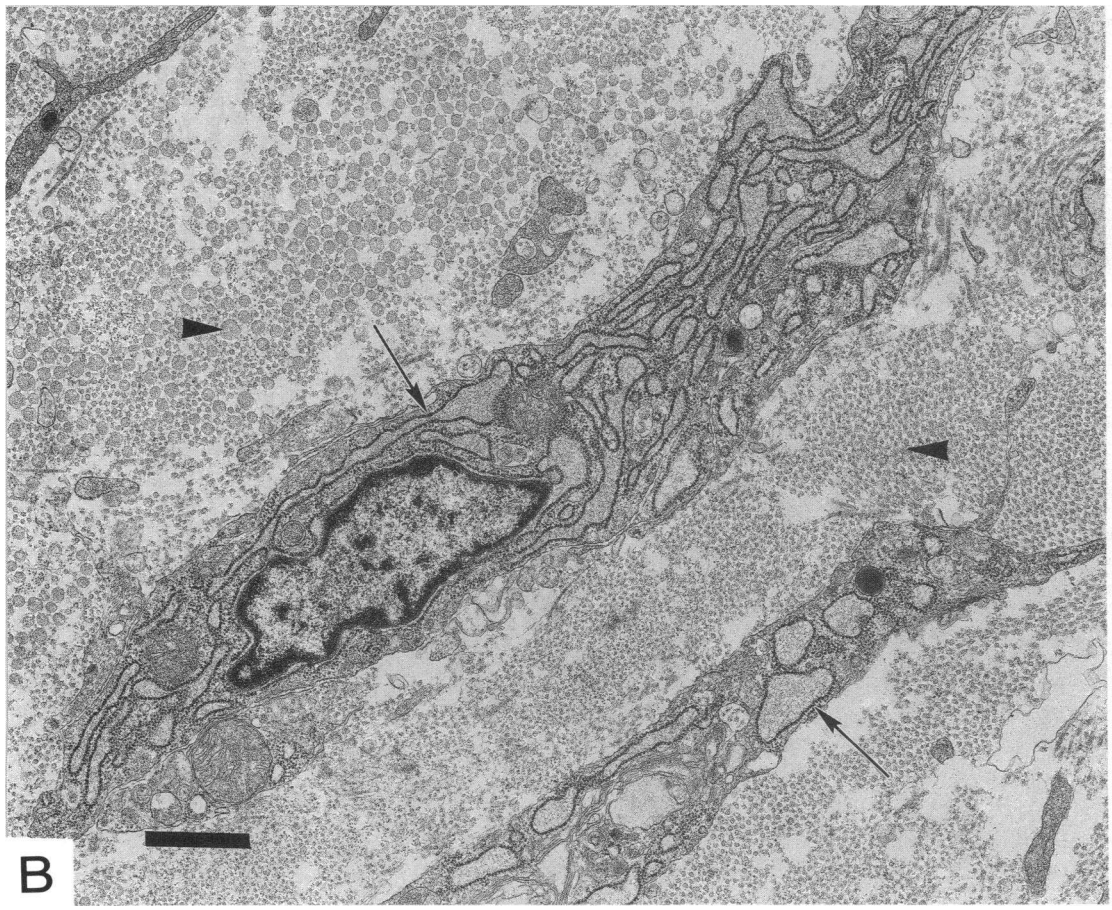
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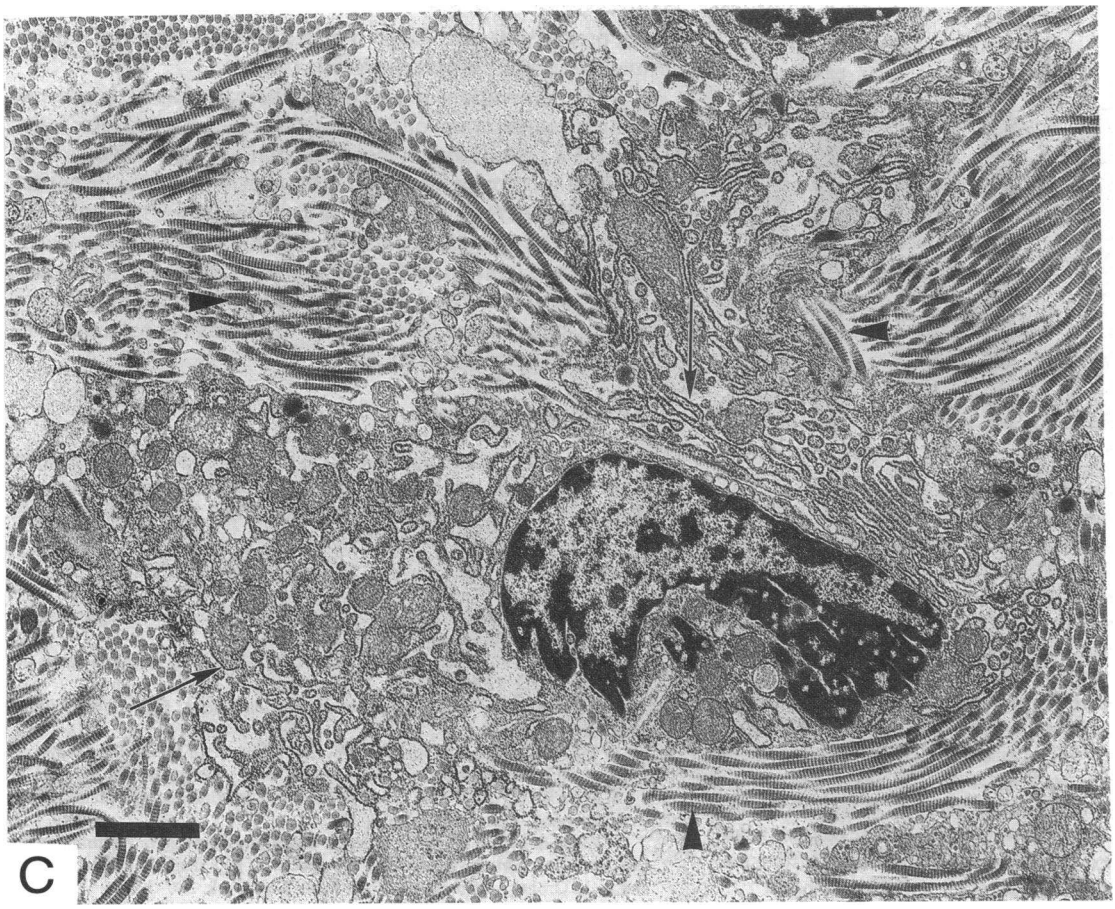

Figure 6. Transmission electron micrographs of incisional wounds. (*A*) Impaired healing control. The extracellular matrix (*arrowheads*) and rough endoplasmic reticulum (*arrows*) of the fibroblasts are not well developed compared with wounds from normal or rhTGF-β1-treated rats, indicating healing processes are impaired (compare with Fig. 5, *B* and *C*). (*B*) Normal healing control. The extracellular matrix (*arrowhead*) is dense and the rough endoplasmic reticulum (*arrows*) within the fibroblasts are well developed, indicating an active secretory phase of wound healing. (*C*) Impaired healing group administered rhTGF-β1 (100 μg/kg). Cell constituents and extracellular matrix are similar to normal healing rats. The extracellular matrix is dense (*arrowhead*) and fibroblasts contain prominent rough endoplasmic reticulum (*arrows*), consistent with an active secretory phase. Samples were obtained 7 d after injury and fixed in Karnovsky's solution (bar = 1 μ).

for wound repair were influenced by the brief systemic exposure to active rhTGF-β1 up to 24 h before injury. Based upon its short half-life and high volume of distribution, it is possible that rhTGF-β1 becomes available to the extravascular environment and thus may be capable of "priming" cells for increased responsiveness to normal regulatory factors released at sites of injury. It has been demonstrated using topically applied [^{125}I]rhTGF-β1 to incisional wounds that 35 and 10% of the applied dose (0.8 mg/kg) was recoverable from the site of the wound at 24 and 48 h, respectively (33a). Binding to a wound site and thus influencing fibroblast responsiveness to secondary signals may be one means by which TGF-β1 enhanced wound strength when administered systemically at the time of injury or 4 h after injury. Enhanced repair when TGF-β1 was administered 24 h before wounding more likely involves "priming" of a circulating cell, most likely the monocyte. Monocytes exposed to active TGF-β1 systemically and having migrated to a wound site would be "primed," i.e., constitutively more active in processes of wound repair and more responsive to other endogenous signals at the wound site.

Recent in vitro observations (34, 35) support this hypothesis. In these studies dermal fibroblasts incubated with TGF-β1 for 24 h increase protein and steady-state messenger RNA levels for collagen and fibronectin as well as for TGF-β1 up to 96 h after removal of exogenous TGF-β1 (34, 35). Primary fetal rat osteoblasts preincubated for 2 h with TGF-β1 followed by incubation with parathyroid hormone or lipopolysaccharide produce more GM-CSF and IL-6 than cells not exposed to TGF-β1 (36). More specifically, monocytes exposed to TGF-β1 increase the response of these cells to secondary stimuli (37). These studies support the hypothesis that TGF-β1 alters cellular responses to subsequent stimuli. The means by which TGF-β1 mediates these changes is unclear but may occur by a variety of means that ultimately lead to enhanced gene expression of additional growth factors and extracellular matrix proteins.

The decreased breaking strength of incisional wounds in our studies of old rats confirms the work of others, in which breaking strength was reduced by ~ 27% compared with the breaking strength of wounds from young adult rats. In addition, our observations are in agreement with others who have demonstrated the stimulatory effect of topically applied TGF-β2 on healing of wounds in old mice (38). Changes associated with aging may also have in vitro counterparts. Dermal fibroblasts in culture exhibit an inverse relationship between the

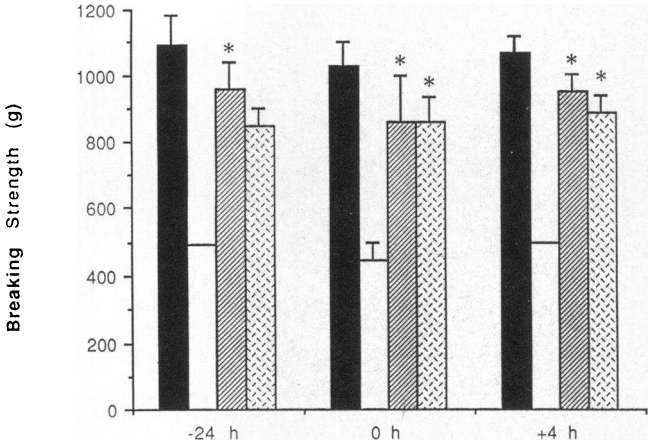

Figure 7. rhTGF-β1 increased breaking strength of wounds of glucocorticoid-impaired rats when administered before, during, or after surgical incision. Breaking strength of incisional wounds was determined from rats administered rhTGF-β1 at 100 μg/kg (▨; n = 2–4) or 500 μg/kg (▧; n = 2–4) either 24 h before (−24 h), 5 min before (0 h), or 4 h after surgery (+4 h). Breaking strength from normal healing rats (■; n = 3–5) and impaired healing rats treated with PBS (□; n = 1–4) were determined concurrently at each time point. Four incisions were placed on each rat as indicated in Methods. Methylprednisolone was administered at the time of injury in all cases except normal healing rats and was independent of administration of rhTGF-β1. The number of rats per group varied between 2 and 4 (8–16 wounds) and was compared with 4 rats (16 wounds) at 0 h and 1 rat (4 wounds) with impaired healing in the −24-h and +4-h groups. These controls were present in three separate experiments and were no different from the 0-h group, i.e., all were impaired with glucocorticoid at 0 h and treated with PBS, with only the injection time of PBS as the variable. The breaking strength means varied no more than 15% in all of our control groups (n = 20). Data represent mean±SEM with significant differences at $^*P < 0.05$.

donor age and the in vitro proliferative capacity (39, 40). This relationship is progressive in rodent fibroblasts up to 12 mo of age, then plateaus and correlates directly with a reduction in healing of the donor site. In addition, motility of human fibroblasts in culture decreases with age of the donor and is independent of chemotactic gradients (41). Others have demonstrated that cytoskeletal (42) as well as genomic changes (43) within fibroblasts correlate with the age of the donor.

Cohen et al. (44) have also demonstrated that a decline in macrophage function, and to a lesser extent number, contributes to a slowing of wound repair as measured by a decrease in wound breaking strength. When antibodies to macrophages are injected into young mice wound healing is retarded. Conversely, wound healing in older mice can be augmented by the injection of autologous macrophages, with an even greater response when macrophages from young mice are administered (45). Additionally, injection of glucan, a known stimulator of macrophages, accelerates wound repair in rodents as measured by an increased wound breaking strength (46).

The systemic administration of TGF-β1 has been shown to modulate other biological processes in vivo as well. Administration of TGF-β1 to rodents undergoing ischemia and reperfusion reduced local circulating superoxide anions and reduced TNF-mediated cellular injury (47, 48). When administered systemically before onset of clinical signs, TGF-β1 prevented the progression of collagen-induced arthritis (49) and experimental allergic encephalomyelitis (50).

The observations reported here indicate that the single systemic administration of rhTGF-β1 can influence cellular functions in a previously unrecognized manner; the active form of TGF-β1 is capable of profoundly altering cellular responses that influence the wound healing cascade. TGF-β1 reversed the healing impairment associated with both age and glucocorticoid administration, a finding that may suggest that healing impairment associated with aging and glucocorticoid administration share a common cellular event that is responsive to growth factor manipulation.

Acknowledgments

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