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Exogenous Transforming Growth Factor-Beta Amplifies Its Own Expression and Induces Scar Formation in a Model of Human Fetal Skin Repair

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Objective

Fetal skin wounds heal without scarring. To determine the role of TGF-β_1 in fetal wound healing, mRNA expression of TGF-β_1 was analyzed in human fetal and adult skin wounds.

Methods

Human fetal skin transplanted to a subcutaneous location on an adult athymic mouse that was subsequently wounded heals without scar, whereas human adult skin heals with scar formation in that location. *In situ* hybridization for TGF-β_1 mRNA expression and species-specific immunohistochemistry for fibroblasts, macrophages, and neutrophils were performed in human adult wounds, fetal wounds, and fetal wounds treated with a TGF-β_1 slow release disk.

Results

Transforming growth factor-β_1 mRNA expression was induced by wounding adult skin. No TGF-β_1 mRNA upregulation was detected in human fetal skin after wounding. However, when exogenous TGF-β_1 was added to human fetal skin, induction of TGF-β_1 mRNA expression in human fetal fibroblasts occurred, an adult-like inflammatory response was detected, and the skin healed with scar formation.

Conclusions

Transforming growth factor-β_1 is an important modulator in scar formation. Anti-TGF-β_1 strategies may promote scarless healing in adult wounds.

Unlike the adult, the wounded midgestational human fetus has the remarkable ability to regenerate normal skin architecture without scarring. Currently, the underlying mechanisms of scarless wound repair remain unknown. Scarless healing may result from a unique cell population, extracellular matrix, or cytokine milieu. The cytokine transforming growth factor-beta (TGF-β) promotes inflammatory cell recruitment and collagen deposition in healing wounds and may play an important role in scar formation. Previous studies on adult rats have shown that wound application of neutralizing antibodies to TGF-β results in markedly reduced scar formation.[1]

Immunostaining for TGF-β_1 protein has demonstrated a deficiency of TGF-β_1 in fetal wounds when compared with adult wounds.[2] Furthermore, addition of TGF-β1 to fetal wounds induces scar formation.[3,4] Human fetal skin transplanted to a subcutaneous position on an adult athymic mouse that is subsequently wounded heals without scar, whereas human adult skin heals with scar formation in that location.[5] By using this model of wound repair, we examined human TGF-β_1 mRNA expression by *in situ* hybridization in adult wounds, fetal wounds, and fetal wounds treated with exogenous TGF-β_1. Species-specific cell types in the wound environment also were determined by immunohistochemistry.

MATERIALS AND METHODS

Animals

Female adult athymic (*nu/nu*) nude mice (Charles River Laboratories, Wilmington, MA) at 6 to 7 weeks of age were housed in groups of four to six in sterile cages at the University of California, San Francisco (UCSF) animal care facility and fed food and water *ad libitum*. Animal management was in accordance with the policies of the UCSF Animal Care Committee and the National Institutes of Health guidelines for the care of experimental animals.

Human Skin

Human fetal scalp skin from 18-week gestational age fetuses (n = 12) was obtained from therapeutic abortion material after signed consent for the use of this tissue for research purposes under an approved UCSF Committee on Human Research protocol. Human fetal tissue collection conformed to the current recommendations of the National Institutes of Health. Gestational age was determined by fetal foot length. Adult human skin (n = 8) from the head and chest was obtained after signed consent from patients undergoing surgery for noninfectious, non-neoplastic conditions. Skin was placed in cooled serum-free Roswell Park Memorial Institute-1640 media (RPMI-1640; Gibco, Grand Island, NY) with 25 mmol/L Hepes, 0.3 g/L L-glutamine, 2.0 g/L NaHCO3, and 1% penicillin/streptomycin. Skin was maintained in these conditions at 4 C for 12 to 24 hours, until transplantation.

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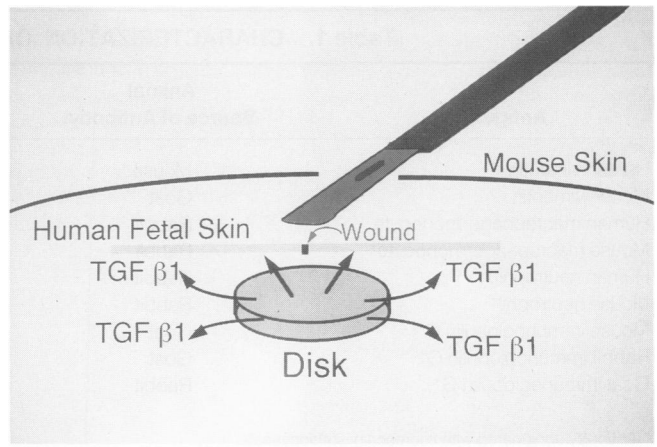

Figure 1. Transforming growth factor-β_1 slow release disks were placed beneath the grafted fetal tissue at the time of wounding.

Transplantation

Skin samples were transplanted onto athymic mice recipients as previously described. Briefly, skin was washed two times in sterile phosphate-buffered saline solution, trimmed into 0.5 cm × 0.5-cm squares, and transplanted. All procedures were performed in a laminar flow hood under aseptic conditions with inhalation ether or metophane anesthesia.

Skin grafts were transplanted into subcutaneous pockets on the flanks of the nude mice. Each mouse received two grafts, one on each flank. The subcutaneous pockets were created by incising a 1-cm length of skin obliquely along the mouse flank and forming a 1-cm × 1-cm cavity underneath the panniculus carnosus. The grafts were placed, with dermis directed down, onto mouse fascia. The pockets were closed with metal clips.

Wounding

Seven days after transplantation, full-thickness linear incisions were made in the grafts. To gain access to the subcutaneous grafts, pockets were opened through a separate incision. Wounds were marked with India ink to aid in localizing the wound during analysis. In one mouse, a cellulose disk designed to release TGF-β_1 over 14 days (Innovative Research, Toledo, OH) was placed directly under the wound, between the human graft's dermis and the mouse's fascia (Fig. 1). The disk placed under the graft in the mouse's right flank contained 10 μg of TGF-β_1 in a cellulose/cholesterol/lactose vehicle. A control disk with an equivalent volume of vehicle was placed under the wounded graft on the mouse's left flank. Mouse skin pockets were closed with clips and the wounds were left to heal.

Table 1. CHARACTERIZATION OF ANTIBODIES USED IN THIS STUDY*

Antigen	Animal Source of Antibody	Manufacturer	Dilution (in Phosphate-Buffered Saline)
Human vimentin	Mouse	Boehringer; Westbury NY	1:200
Mouse vimentin	Goat	ICN; Costa Mesa CA	1:200
Human macrophage/monocyte	Rabbit	Chemicon; Temcula, CA	1:200
Mouse macrophage/monocyte*	Rabbit	Accurate; Westbury, NY	1:1000
Human neutrophil	Rabbit	Chemicon; Temcula, CA	1:200
Mouse neutrophil*	Rabbit	Accurate; Westbury, NY	1:1000
Mouse immunoglobulin G*	Goat	Sigma; St. Louis, MO	1:250
Rabbit immunoglobulin G*	Goat	Sigma; St. Louis MO	1:80
Goat immunoglobulin G*	Rabbit	Sigma; St. Louis, MO	1:80

* Antibody conjugated with fluorescein isothiocyanate.

Harvesting of Grafts

The mice were killed 1, 6, and 12 hours and 1, 3, 7, and 14 days after wounding, and the grafts were removed. Grafts with the slow-release disks were harvested 14 days after wounding. Wounded fetal skin grafts were snap-frozen using precooled isopentane in liquid nitrogen. Wounds were cut in 8-μm sections on a Reichert-Jung cryostat at −20 C and mounted on polysialic acid glass slides in preparation for *in situ* hybridization and immunostaining. All tissue and slides were stored at −80 C.

Preparation of Probes

The human TGF-β_1 sequence was compared with GenBank, and specific sequences for human TGF-β_1 were isolated. These sequences were then compared with the mouse TGF-β_1 sequence. Human-specific TGF-β_1 sequences were selected to avoid cross-hybridization with mouse TGF-β_1. Antisense oligonucleotide probes (one 26 base-pair sequence from exon 7, one 27 base-pair sequence from exon 7, and one 28 base-pair sequence from exon 6) were synthesized (R&D Systems, Minneapolis, MN) and end-labeled with biotin. Probes were purified by polyacrylamide gel electrophoresis and supplied in an equimolar mixture.

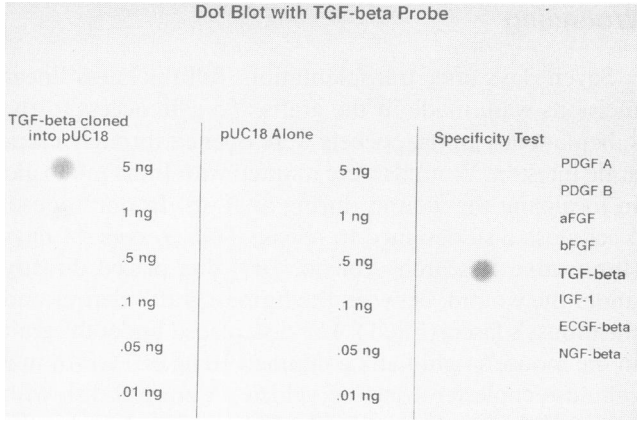

Figure 2. Dot blot of human TGF-β_1 probe against 0.01–5.0 ng of TGF-β_1 cloned into pUC18, against 0.01–5.0 ng of pUC18 alone, and against 5 ng of PDGF A, PDGF B, aFGF, bFGF, TGF-β, IGF-1, ECGFβ, and NGF-β cloned into pUC18.

Probe Specificity

Dot-blot hybridization was performed to determine specificity and sensitivity of the probe, using standard techniques.[6] The human-specific TGF-β_1 probes from R&D Systems were hybridized against 0.01 to 5 ng of TGF-β_1 cloned into the pUC18 plasmid to determine sensitivity of the probe for TGF-β_1, against 0.01 to 5 ng of pUC18 alone to rule out the possibility of the probe actually detecting the pUC18 plasmid but not TGF-β_1, and against 5 ng of platelet-derived growth factor A (PDGF A), platelet-derived growth factor B (PDGF B), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), TGF-β, insulin-like growth factor 1 (IGF-1), endothelial-cell growth factor beta (ECGF-β), and nerve growth factor beta (NGF-β) cloned into pUC18 to determine the sensitivity of the probe. Filters were washed twice for 15 minutes at 42 C and exposed for 3 days.

In Situ Hybridization

Slides were warmed to room temperature in a moist chamber. The slides were successively washed in phosphate-buffered saline (2 × 5 minutes, 20 C), 2X SSC (10 minutes, 60 C), H_2O (rinse, 20 C), 50 mmol/L Tris pH 7.6 (5 minutes, 20 C), and then incubated in 5 μg of proteinase K (Sigma, St. Louis, MO) per milliliter of 0.05 M

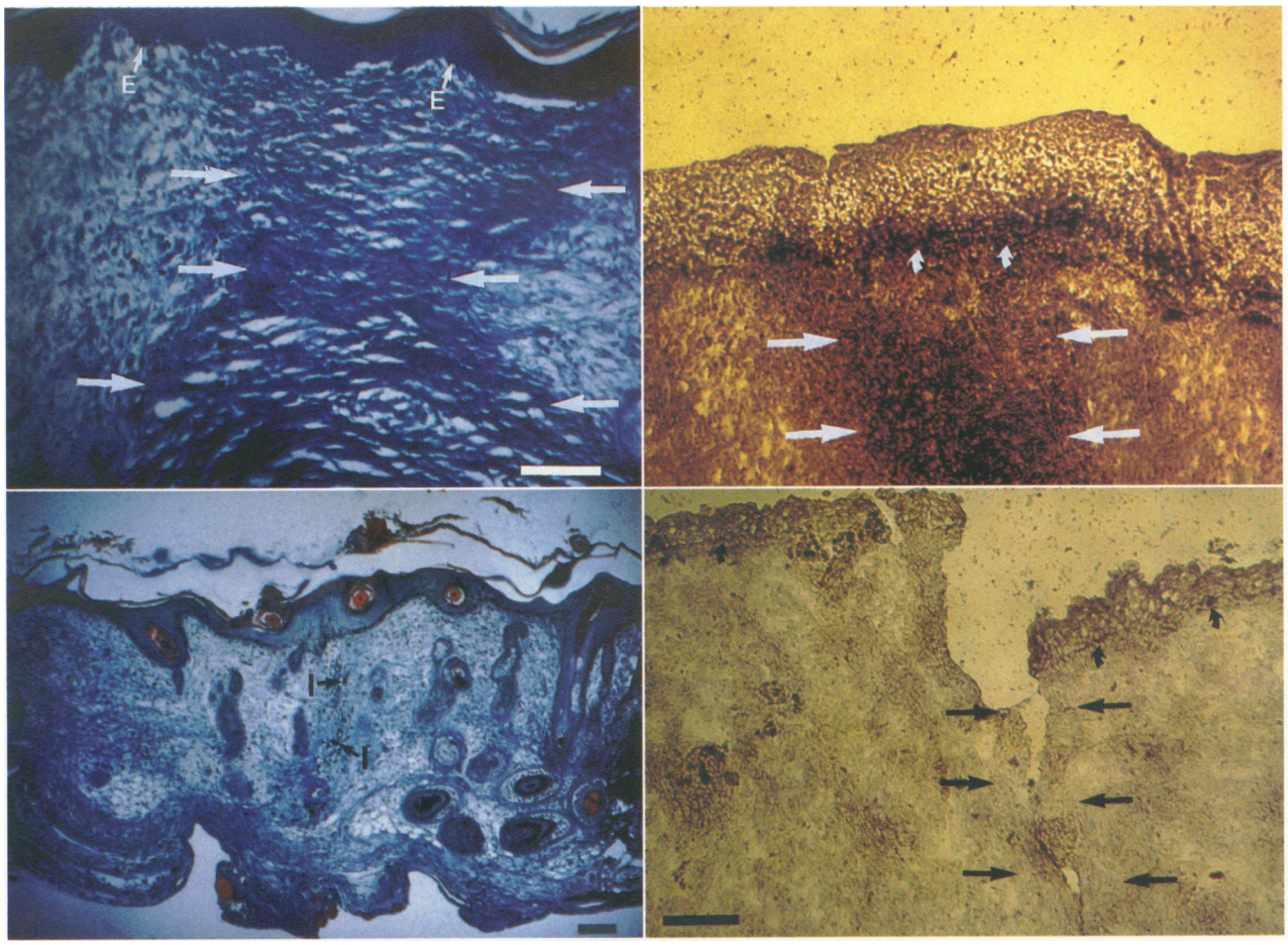

Figure 3. (A) (*top left*) Trichrome histology of a healed human adult skin graft placed in the subcutaneous position of the nude mouse. Trichrome stains collagen a deep blue. The adult skin heals with disorganized collagen formation characteristic of scar (arrows). (B) (*top right*) *In situ* hybridization for TGF-β_1 mRNA was performed on adult skin 7 days after wounding. Cells producing TGF-β_1 mRNA are found throughout the wound (straight arrows) and in the basal layer of the epidermis (curved arrows) (E = epidermis; scale bar = 100 μm)

Figure 4. (A) (*bottom left*) Trichrome histology demonstrates that the human fetal skin healed without scar. India ink (I) demarcates the initial site of injury. (B) (*bottom right*) *In situ* hybridization for TGF-β_1 mRNA was performed on fetal skin 3 days after wounding. Transforming growth factor-β_1 was detected in the basal layer of the epidermis (curved arrows). General background staining was seen in the dermis but no cells producing TGF-β_1 mRNA were found (scale bar = 100 μm).

Tris pH 7.6 at 37 C for 1 hour. Sections were then rinsed in 1X phosphate-buffered saline, incubated in 4% paraformaldehyde in 1X phosphate-buffered saline at 4 C for 20 minutes, and then rinsed in H_20. Slides were prehybridized with 30% (v/v) formamide, 4X SSC, and 150 μg/mL sonicated salmon sperm DNA for 1 hour at 37 C in a humid hybridization chamber and then hybridized in a solution containing 30% (v/v) formamide, 4X SSC, 10% (w/v) dextran sulfate, 150 μg/mL of sonicated salmon sperm DNA, and 200 ng/mL of the probe for 24 hours at 37 C in a moist chamber. After hybridization, the sections were successively washed in 4X SSC/30% (v/v) formamide (2 × 5 minutes, 37 C), 2X SSC/30% (v/v) formamide (2 × 5 minutes, 37 C), and 0.2X SSC/30% (v/v) formamide (2 × 5 minutes, 37 C). To demonstrate that the target nucleic acid was RNA, control tissue sections were pretreated with 100 μg of RNase/mL of 2X SSC and 10 mmol/L $MgCl_2$ for 1 hour at 37 C before hybridization.

Detection of Biotin-Labeled Probes

Tissue sections were blocked for 30 minutes with a solution containing 3% protease-free bovine serum albumin (Sigma), 0.1 mol/L Tris-Cl pH 7.5, 0.2 mol/L NaCl, and 0.05% Triton-X-100 (Sigma). Slides were then incu-

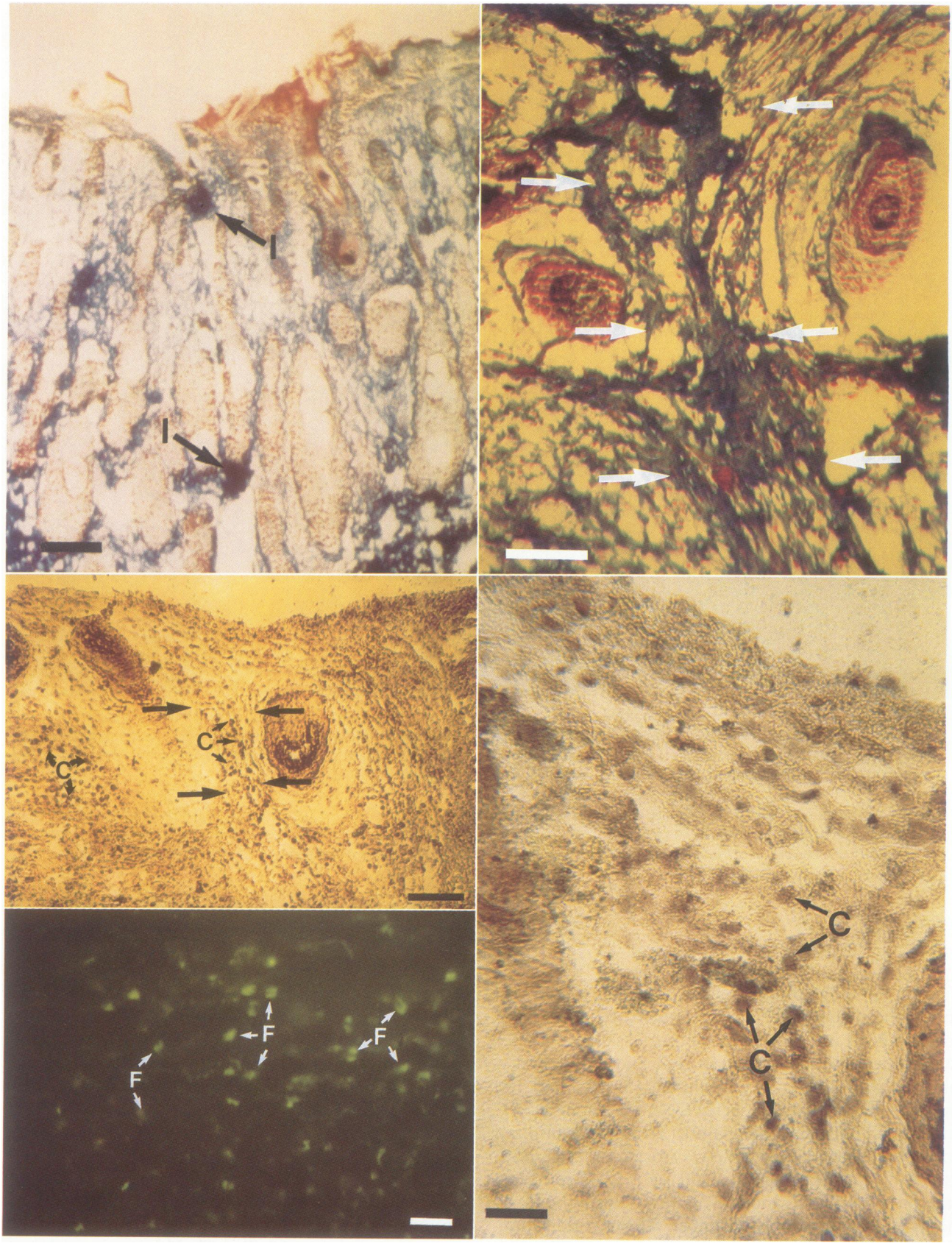
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Figure 5. (A) (*top left*) Trichrome histology of the fetal graft with the control disk demonstrates the typical scarless healing seen in fetal skin. No disorganized collagen formation or scar is seen. India ink (I) particles mark the wound site. (B) (*top right*) Trichrome histology of the wounded fetal skin graft with a 10-μg slow release TGF-β_1 disk demonstrates a disorganized collagen pattern characteristic of scar (arrows). *In situ* hybridization for TGF-β_1 mRNA on a serial section from the fetal skin with the TGF-β_1 slow release disk was then performed (C) (*middle left*). The area of scar is denoted by the large arrows. Cells producing TGF-β_1 mRNA (C) are found throughout the wound (indicated by small arrows) and the surrounding unwounded dermis (indicated by curved arrows). (D) (*bottom right*) Under higher magnification at the scar site, cells producing TGF-β_1 mRNA (C) are seen. (E) (*bottom left*) Immunohistochemistry for human vimentin showed that these cells are human fibroblasts (F). (5A–5C: scale bar = 100 μm; 5D–5E: scale bar = 30 μm)

bated in 10 μg of streptavidin alkaline phosphatase per milliliter of 0.1 mol/L Tris-Cl pH 7.5, 0.2 mol/L NaCl, and 0.05% Triton-X-100 for 25 minutes. Three 10 minutes washes in 0.1 mol/L Tris-Cl pH 7.5, 0.2 mol/L NaCl, and 0.05% Triton-X-100 were followed by a 10-minute wash in 0.1 mol/L Tris-Cl pH 9.5 and 0.1 mol/L NaCl. The color development was carried out by incubating the slides for 30 minutes at room temperature in a substrate solution containing 0.66 mg/mL of nitroblue tetrazolium and 0.33 mg/mL of 5-bromo-4-chloro-indolyl phosphate in 0.1 mol/L Tris-Cl pH 9.5 and 0.1 mol/L NaCl. The reaction was stopped by incubating the slide in 1 mmol/L of edetic acid.

Immunohistochemistry

Primary antibodies used were specific for human and mouse vimentin (a fibroblast marker), human and mouse macrophages, and human and mouse neutrophils (Table 1). Sections were fixed in acetone for 1 to 2 minutes at room temperature. Serum from the species of the primary and secondary antibody was used to block nonspecific background binding. Sections were stained with primary antibody for 1 hour, washed with phosphate-buffered saline, and stained with fluorescein-linked immunoglobulin G secondary antibody. Sections were viewed and photographed with a Zeiss fluorescent microscope (Zeiss, Berlin, Germany). Negative control sections had nonimmune goat serum (Cedarlane Laboratories Limited, Hornby, Canada) substituted for primary antibody. Mouse and human dermis sections that were not wounded were stained separately with their corresponding species-specific primary antibody and counter-specific antibody to function as both positive and negative controls.

RESULTS

Dot-blot hybridization demonstrated that the TGF-β_1 probe was able to detect up to 0.05 ng/ml of TGF-β_1 cloned into pUC18. The TGF-β_1 probe did not detect the pUC18 plasmid alone. The TGF-β_1 probe hybridized with the cDNA of TGF-β_1, but did not hybridize with

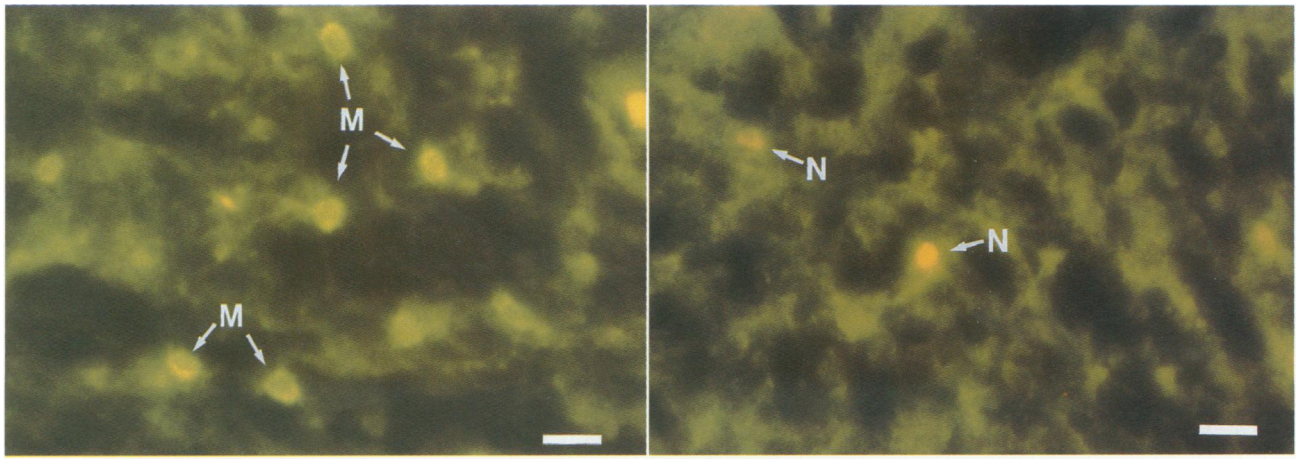

Figure 6. (A) (*left*) Immunohistochemistry with species-specific antibodies demonstrated an influx of mouse macrophages (M) into the wound. (B) (*right*) Immunohistochemistry also revealed a recruitment of mouse neutrophils (N) into the wound. No human macrophages, human neutrophils, or mouse fibroblasts were detected in the wound. (scale bar = 30 μm)

the cDNA of PDGF A, PDGF B, aFGF, bFGF, IGF-1, ECGF-β, or NGF-β cloned into pUC18 (Fig. 2).

Adult grafts from 1 (n = 4), 6 (n = 4), and 12 (n = 4) hours and 1 (n = 4), 3 (n = 4), 7 (n = 4), and 14 (n = 4) days after wounding were analyzed. Hematoxylin and eosin (H&E) and trichrome histology showed that the adult skin healed with scarring (Fig. 3A). Transforming growth factor-β_1 mRNA was detected in the basal layer of the epidermis and in the hair follicles of all adult grafts. It also was detected in the dermis of adult wounds at 1, 3, and 7 days postwounding (Fig. 3B). Human fibroblasts and human macrophages were found in the wound by immunohistochemistry.

Fetal grafts from 1 (n = 4), 6 (n = 4), and 12 (n = 4) hours and 1 (n = 4), 3 (n = 4), 7 (n = 4), and 14 (n = 4) days after wounding were analyzed. Hematoxylin and eosin and trichrome histology showed that the fetal skin healed without scarring (Fig. 4A). Transforming growth factor-β_1 mRNA was detected in the basal layer of the epidermis and in the hair follicles of all fetal grafts and served as a positive control. No TGF-β_1 mRNA was detected in the dermis of fetal wounds at any time point (Fig. 4B). Immunohistochemistry demonstrated exclusively human fibroblasts in the subcutaneous graft. No human macrophages, human neutrophils, mouse macrophages, or mouse neutrophils were found.

Fetal grafts with the slow release disks implanted underneath the graft were analyzed 14 days after wounding. Trichrome histology showed that the graft above the control disk healed without scar (Fig. 5A), whereas the graft above the TGF-β_1 disk healed with the disorganized collagen formation characteristic of scar (Fig. 5B). *In situ* hybridization with human-specific TGF-β_1 probe demonstrated marked induction of TGF-β_1 mRNA in wounds treated with the slow release disks of TGF-β_1 (Figs. 5C and 5D). On serial section, immunohistochemistry revealed that the cells with TGF-β_1 expression were human fetal fibroblasts (Fig. 6A). Adult mouse macrophages (Fig. 6B) and adult mouse neutrophils (Fig. 6C) also were detected at the wound site, but no human macrophages or human neutrophils were found. When sections were pretreated with RNase before *in situ* hybridization, no TGF-β_1 mRNA was detected. No TGF-β_1 induction was seen in the fetal grafts with the control disks.

DISCUSSION

Human fetal skin transplanted onto nude mice in the subcutaneous position has been shown to be a novel model of human fetal wound repair. These grafts retain all the morphologic and ultrastructural features associated with normal fetal skin development.[7] *In situ* hybridization studies with species-specific probes and immunohistochemistry have demonstrated that human fetal cells are the predominant cells involved in wound repair in the subcutaneous grafts. No significant murine interaction was found in the healing of subcutaneous wounds.[8,9]

In this experimental system, wounding adult skin induces TGF-β_1 mRNA expression whereas wounding fetal skin does not.However, if exogenous TGF-β_1 protein is added to human fetal skin at the time of wounding, human fetal fibroblasts are induced to produce TGF-β_1 mRNA, adult mouse macrophages and adult mouse neutrophils are recruited to the wound, and the wound heals with scarring.

Of the many cytokines implicated in wound healing, TGF-β affects all phases of the healing process, including the inflammatory response and matrix accumulation.[10] The mammalian TGF-β family consists of three known isoforms, TGF-β_1, TGF-β_2, and TGF-β_3, which are structurally and functionally related to one another. Transforming growth factor-β_1 is a disulfide-linked dimer of two identical chains of 112 amino acids.[11] Each chain is synthesized with a 390 amino acid C-terminal precursor. On secretion, the C-terminal precursor region and a TGF-β_1 binding protein form a complex that keeps the cytokine inactive.[12] Active TGF-β_1 can be released from the latent complex by exposure to extreme pH or plasmin.[13] Through autocrine and paracrine mechanisms, TGF-β_1 stimulates the deposition of collagen and other matrix components by fibroblasts, inhibits collagenase, blocks plasminogen inhibitor, enhances angiogenesis, and is chemotactic for fibroblasts, monocytes, and macrophages.[14] Thus, TGF-β_1 plays a central role in the initiation of fibrosis.

Early gestation human fetal skin wounds appear to be TGF-β_1 deficient. Immunohistochemical studies have revealed an absence of both latent TGF-β_1 and active TGF-β_1 protein in fetal skin when compared with adult wounds.[4] Our *in situ* hybridization results parallel those obtained by immunostaining for TGF-β_1 protein. These studies demonstrate that wounding fetal skin does not induce expression of either TGF-β_1 protein or TGF-β_1 mRNA. Transforming growth factor-β_1 levels in fetal wounds may be below the threshold for detection by immunohistochemistry or *in situ* hybridization. However, more sensitive methodologies, such as reverse transcriptase polymerase chain reaction, require tissue homogenates. These results would be difficult to interpret because fetal skin constitutively expresses TGF-β_1 in the basal layer of the epidermis and in the hair follicles. *In situ* hybridization with biotin-labeled oligonucleotide probes allows for the detection of the actual mRNA producing cells on tissue section and still is quite sensitive. Proponents of *in situ* hybridization have advocated that this method can detect as few as five mRNA transcripts per cell and that biotin-labeled oligonucleotide probes are as sensitive as radio-labeled probes.[15,16] Thus, compared with scarring adult wounds, nonscarring fetal

wounds are at least relatively TGF-β_1 protein and mRNA deficient.

In vivo studies have demonstrated that exogenously applied TGF-β promotes scar formation and increases collagen deposition in both adult and fetal wounds. In adult wound models, low-dose subcutaneous injection of TGF-β_1 stimulates fibrosis and accumulation of fibroblasts, macrophages, and granulocytes.[17] Furthermore, wound repair studies using subcutaneous chambers have demonstrated that TGF-β_1 induces rapid formation of connective tissue.[14] In fetal wounds, the addition of TGF-β to polyvinyl alcohol sponges implanted in fetal rabbits produces fibrosis,[3] and TGF-β_1 slow release disks induce dose-dependent scarring in human fetal skin after wounding.[4] *In vitro* studies have shown that TGF-β_1 enhances the synthesis of collagen, fibronectin, elastin, hyaluronate, and other matrix components.[18–20] Exposure of fetal dermal fibroblasts to TGF-β_1 results in marked upregulation of collagen gene expression.[21] Thus, the cellular and matrix machinery that is necessary for scar formation exists in fetal wounds.

The cellular response to TGF-β_1 administration during wound repair is unclear. *In situ* hybridization studies of an adult pig skin excisional model demonstrated that TGF-β_1 injections enhance mRNA content of collagen type I, collagen type III, fibronectin, and TGF-β_1 itself while stromelysin mRNA expression decreases.[22] Our study shows that exogenous TGF-β_1 actually induces human fetal fibroblasts to produce TGF-β_1 mRNA at the wound site and in the surrounding dermis. *In vitro* studies have demonstrated that TGF-β_1 enhances transcription of its own mRNA and thus amplifies its effects.[23] Mediators released from inflammatory cells also have been shown to increase TGF-β_1 expression in adult fibroblasts.[14,21] Thus, the induction of TGF-β_1 mRNA in fetal fibroblasts is probably the result of both the direct effect of exogenous TGF-β_1 and the recruitment of inflammatory cells with their subsequent release of cytokines. Fetal fibroblasts have also been shown *in vitro* to be capable of producing TGF-β_1.[24] The presence of "activated" TGF-β_1 cells in the wound site appears to be important for the induction of scarring in fetal skin. Transforming growth factor-β_1 may have induced the fetal fibroblasts to become more adult-like.

Recent fetal wound healing studies have correlated the absence of scarring with a sparse inflammatory response, as evidenced by markedly reduced neutrophil, macrophage, and monocyte infiltrates,[25,26] absence of endogenous immunoglobulins at the wound site,[27,28] reduced angiogenesis, and altered levels of peptide growth factors.[29] The transition of the fetal healing phenotype to a scarring adult phenotype in the marsupial correlates directly with the amount of inflammatory reaction at the wound site.[30] Our study demonstrates that, in the subcutaneous fetal graft wounds, there is a lack of an inflammatory cell infiltrate during scarless fetal repair. However, exogenously added TGF-β_1 during wounding promotes an adult-like inflammatory response, and the wound heals with scarring. The presence of adult mouse neutrophils and adult mouse macrophages that are recruited to the subcutaneous human fetal graft wound may be another reason why scar formation occurs.

Strategies to block TGF-β may prevent scarring. Injection of anti-TGF-β polyclonal neutralizing antibodies into adult rat wounds markedly diminishes scarring.[1] Subsequent studies have revealed that specific neutralization of both TGF-β_1 and TGF-β_2 isoforms has a much greater synergistic antiscarring effect than neutralization of either isoform alone.[31] Injection of the TGF-β_3 isoform at the time of wounding downregulates TGF-β_1 and TGF-β_2 levels and leads to a pronounced antiscarring result.[32] Not only do anti-TGF-β strategies seem effective in reducing scar formation in adult wounds, they also may be effective in ameliorating the effects of other fibrotic processes. For instance, administration of either TGF-β_1 antiserum or decorin (a polysaccharide that binds and inactivates TGF-β_1) suppresses the pathological increase in matrix synthesis that occurs in an animal model of glomerulonephritis.[33,34]

Future fetal wound healing studies will help us understand the biology of scarless healing and may yield insight into the prevention of scar formation. Recent advances in fetal wound healing research suggest a number of ways in which the matrix and cellular response of the healing adult wound might be manipulated to reduce scarring.[35] Rigorous testing of anti-TGF-β strategies may lead to clinical induction of scarless healing in adults and children.

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