A Model of Scarless Human Fetal Wound Repair is Deficient in Transforming Growth Factor Beta

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● **Human fetal skin heals via scarless regeneration, whereas adult skin heals with scar. Scarless repair may reflect a distinct cytokine milieu. We studied the role of the cytokine transforming growth factor beta (TGFβ) using an established model of scarless human fetal skin repair in which human fetal skin is transplanted into a subcutaneous pocket on the flank of an adult nude mouse. In this model, wounded 16-week-gestation human fetal skin heals without scar, whereas wounded adult skin heals with scar. Seven days after transplantation, incisional wounds were made in the skin grafts. In the first phase of the study, wounds were harvested from 1 hour to 4 weeks postwounding, and immunohistochemistry was performed for TGFβ (isoform nonspecific), $TGF\beta_1$, and $TGF\beta_2$. Scarfree wounds in the fetal skin grafts did not show TGFβ staining. In contrast, wounds in adult grafts that heal with scar demonstrated isoform nonspecific TGFβ staining from 6 hours through 21 days, $TGF\beta_1$ from 6 hours through 21 days, and $TGF\beta_2$ from 12 hours through 7 days. In the second phase of the study, a slow-release disk with 0.01, 0.1, 1.0, or 10 μg of $TGF\beta_1$ was placed beneath the fetal skin graft at the time of wounding. Fourteen days postwounding, there was marked scarring in the fetal grafts treated with $TGF\beta_1$, and the size of the scar was proportional to the amount of $TGF\beta_1$ applied. The relative lack of TGFβ, a cytokine known to promote fibrosis, may be one reason why the fetus heals by regeneration rather than scarring. In contrast, the fibrosis characteristic of postnatal wound repair may be associated with an excess of TGFβ. These findings suggest that anti-TGFβ therapeutic strategies may ameliorate scar formation in children and adults.**
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UNLIKE ADULT SKIN, human fetal skin has the remarkable ability to heal by scarless regeneration.[1] The mechanisms underlying this difference are unknown, but may reflect a different cell population, extracellular matrix, or cytokine profile. The cytokine transforming growth factor beta (TGFβ) promotes inflammatory cell recruitment and collagen deposition in healing postnatal wounds,[2] and exogenously applied TGFβ promotes fibrosis in fetal rabbit wounds.[3] We hypothesized that scarless human fetal wounds are TGFβ-deficient and that adding TGFβ to human fetal wounds would cause scarring. To test this hypothesis, we used a human wound healing model in which adult and fetal human skin grafts were transplanted onto adult athymic nude mice and then wounded.[4] To detect TGFβ in the wounds, grafts were immunostained for TGFβ and for the specific isoforms $TGF\beta_1$ and $TGF\beta_2$. Exogenous $TGF\beta_1$ was added to a second set of fetal wounds to study the effect of this cytokine on scarless human fetal healing.

MATERIALS AND METHODS

Animals

Six- to 7-week-old female adult athymic (nu/nu) nude mice (Charles River Laboratories, Wilmington, MA) were housed in groups of five in sterile cages at the University of California, San Francisco, and fed food and water ad libitum.

Skin

Human fetal skin samples from the head and neck were obtained from therapeutic abortion material after obtaining signed consent for the use of this tissue for research purposes under a protocol approved by the University of California, San Francisco, Committee on Human Research. Human fetal tissue collection conformed to the current recommendations of the National Institutes of Health. Gestational age was determined by fetal foot length. Postnatal human skin from the head (42 year old) and chest (34 year old) was obtained after signed consent from patients undergoing plastic surgery for noninfectious nonneoplastic conditions. The 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 $NaHCO_3$, and 1% penicillin/streptomycin. The skin was stored at 5°C for 1 to 6 hours, until transplantation.

Transplantation

The skin samples were washed two times in sequential phosphate-buffered solutions (University of California, San Francisco, Cell Culture Facility), trimmed of subcutaneous fat, cut into 0.5- × 0.5-cm squares, and transplanted (Fig 1). All procedures were performed in a laminar flow hood, under aseptic conditions, with general methoxyflurane anesthesia (Metofane; Pitman-Moore, Mundelein, IL).

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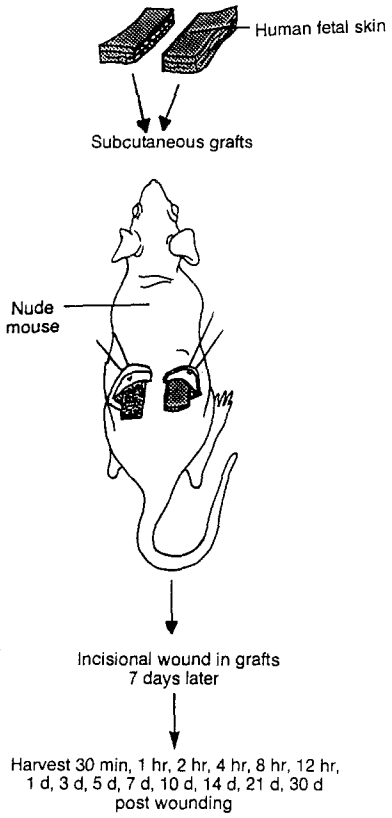

Fig 1. Schematic of the experimental algorithm of human fetal skin transplantation onto the nude mouse. Each mouse received either two fetal or two adult subcutaneous grafts in the dorsolateral flank locations.

The grafts were placed into a subcutaneous pocket on the mouse's flank. The pocket was created by incising a 1 cm of skin obliquely, along the flank, and raising a full-thickness flap to excavate a 1- × 1-cm^2 cavity. The mouse panniculus carnosus was raised from the underlying fascia, and the human skin grafts were positioned such that graft dermis apposed the mouse fascia. Each mouse received two grafts. After graft placement, the mouse's skin incisions were closed with surgical clips, and graft healing occurred in an airtight pocket.

Wounding

Seven days after transplantation, the subcutaneous pockets were reopened and the grafts exposed. Linear incisions were made through the grafts. The incisional wounds were marked with India ink to facilitate wound localization during histological analysis. At this point, the pockets on the flanks of mice in the first phase of the study were closed with clips, and graft wounds were left to heal. For each of four mice in the second phase of the study, a cellulose pellet designed to release $TGF\beta_1$ over 14 days (Innovative Research, Toledo, OH) was placed in the area directly under the wound, between the human graft's dermis and the mouse's fascia (Fig 2). The disk placed under the graft in the mouse's right flank contained either 0.01, 0.1, 1.0, or 10 μg of $TGF\beta_1$ in a cellulose/cholesterol/lactose vehicle. An identical disk with an equivalent volume of vehicle was placed under the wounded graft in the mouse's left flank. After disk placement, the mouse skin pockets were closed with clips, and the wounds were left to heal.

Wound Harvest and Processing

For phase 1 grafts, wounds were harvested at hours 1, 6, 12, 18, 24, 36, 48, 72, and 120, and weeks 1, 2, 3, and 4 postwounding. The animals were euthanized by ether anesthesia and bilateral thoracotomy. The wounded grafts were snap-frozen in precooled isopentane, stored at −80°C, and cut into 6-μm sections on a Reichart-Jung cryostat (Leica, Deerfield, IL). The sections were treated with antibody to TGFβ (isoform nonspecific), $TGF\beta_1$, and $TGF\beta_2$ (R&D Systems, Minneapolis, MN). Additional sections were treated with antihuman latency-associated polypeptide (LAP) $TGF\beta_1$ detection antibody (R&D Systems) to determine whether the biologically inactive precursor form of $TGF\beta_1$ was present. Secondary antibodies were labeled with fluorescein (Sigma, St Louis, MO). Eight hundred sections from 60 wounded grafts of 30 mice were examined and photographed under a Zeiss fluorescent microscope. For phase 2 of the study, the grafts were processed through serial alcohol dehydrations with a toluene final step, were paraffin-embedded, and were cut to 10 μm on an AO-820 microtome (American Optical, Buffalo, NY). The sections were stained with H&E and Mallory's trichrome.

RESULTS

Wounds in the human fetal skin grafts demonstrated an overall absence of TGFβ, $TGF\beta_1$, and $TGF\beta_2$ (Fig 3A). The only exceptions were two of the 20 samples harvested at 48 and 72 hours postwounding, which stained very faintly for $TGF\beta_1$ at the wound edge. $TGF\beta_1$ staining was not present at other time-points. Brightly stained hair follicles served as the positive control, because TGFβ, $TGF\beta_1$, and $TGF\beta_2$ were present in the hair follicles of normal unwounded fetal and adult skin. In contrast, wounds in adult grafts demonstrated isoform nonspecific TGFβ from 6 hours through 21 days, $TGF\beta_1$ at the wound edge from 6 hours through 21 days, and $TGF\beta_2$ from 12 hours through 7 days (Fig 3B). $TGF\beta_1$ LAP was not detected at the wound edge in fetal grafts but was detected in adult wounds from 6 through 18 hours postwounding (data not shown).

In the second phase of the study, there was marked scarring in the fetal grafts, above the $TGF\beta_1$ slow-

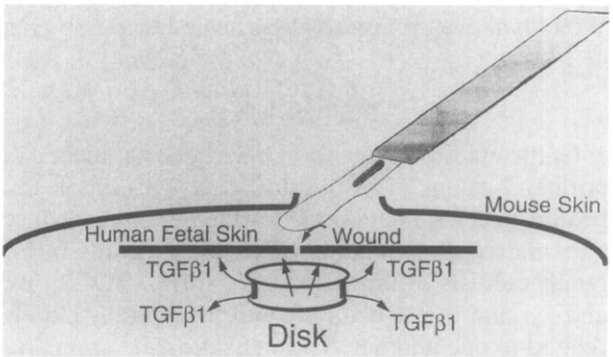

Fig 2. Schematic of the slow-release TGFβ disk underneath the wounded human fetal skin graft in the subcutaneous position on the adult nude mouse flank.

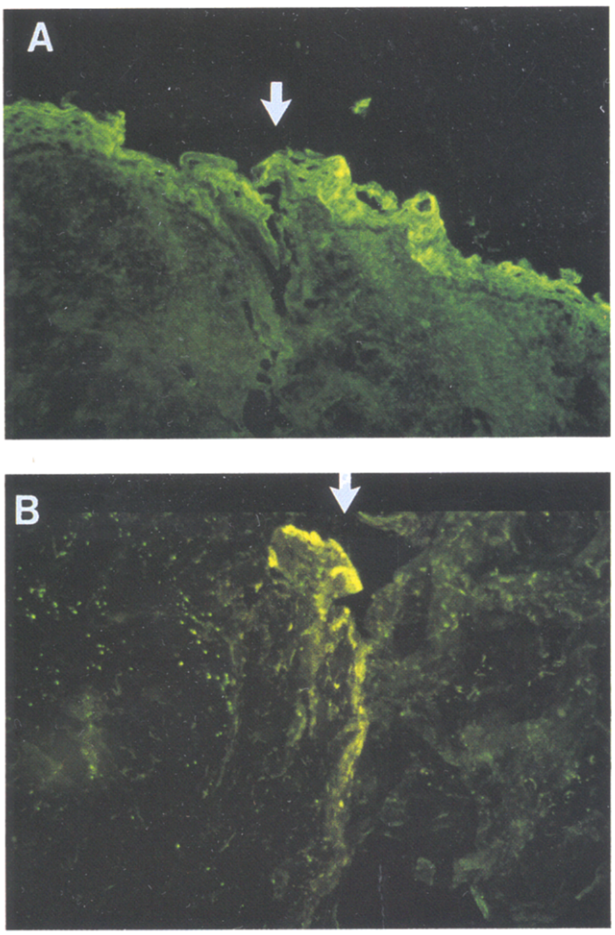

Fig 3. (A) Sixteen-week fetal skin graft immunostained for TGFβ_2 4 hours postwounding. The epidermis stains positively for TGFβ_2 and is seen at the green border at the upper edge of the graft. The wound (arrow) does not stain for TGFβ_2. (B) Adult skin graft immunostained for TGFβ, isoform nonspecific, 12 hours postwounding. The epidermis is out of view. The wound (arrow) stains for TGFβ.

release disks (Fig 4A). The degree of fibrosis in these wounds was minimal with the 0.01-μg concentration and became more pronounced with increasing doses. Wounds above the control disks healed scarlessly (Fig 4B).

DISCUSSION

Of the many cytokines that have been implicated in normal healing, TGFβ affects all phases of the healing process, including the inflammatory response and matrix accumulation. The mammalian TGFβ family consists of three known isoforms, TGFβ_1, β_2, and β_3, that are structurally and functionally closely related to one another. Through autocrine and paracrine mechanisms, TGFβ 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.[5] Thus, TGFβ is capable of stimulating fibrogenesis by the adult fibroblast.

This study shows that there is a deficiency of TGFβ in scarless human fetal wounds, and that adding TGFβ_1 to human fetal wounds promotes scarring. In the first phase of our study, immunohistochemistry detected a lack of TGFβ in 16-week-gestation human fetal wounds. This finding correlates with a study of mouse lip wounds, in which TGFβ was present in

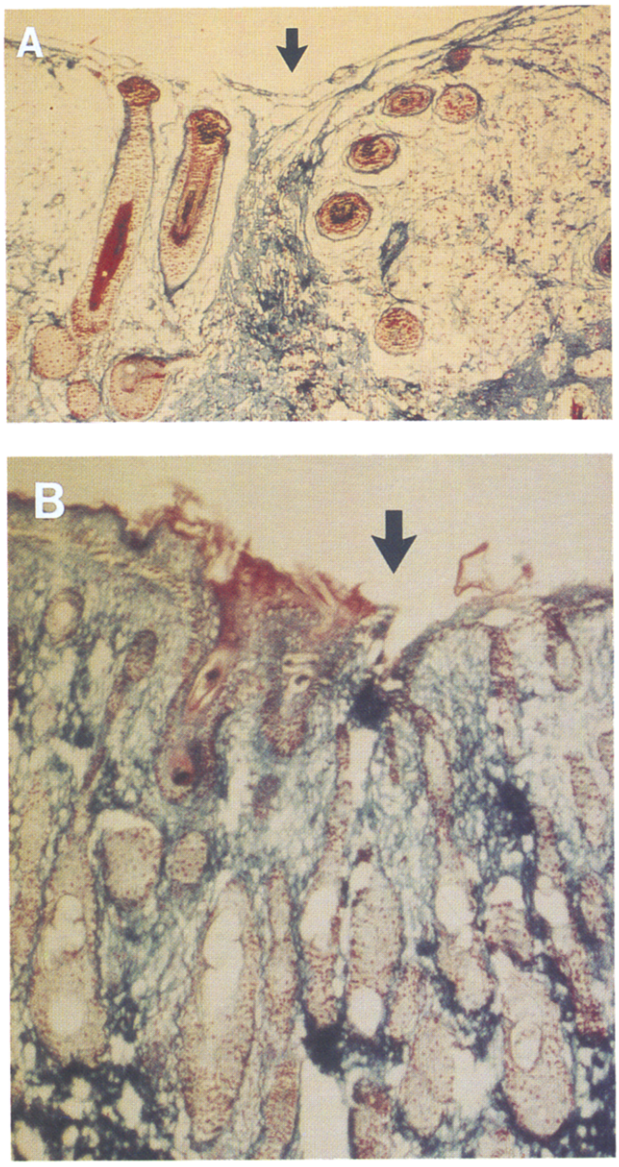

Fig 4. Mallory's trichrome-stained sections of 16-week fetal skin graft, 14 days postwounding (A) Placement of disk containing 10 μg of TGFβ_1. Note blue staining of collagen scar at the wound (arrow). (B) Placement of control disk. Arrow indicates the location of the scarless wound.

neonatal and adult wounds but not in fetal wounds.[6] Similarly, Nath et al showed that $TGF\beta_1$ and $TGF\beta_2$ protein were detected immunohistochemically in the normal developing fetal rabbit dermis, but were not upregulated at the fetal rabbit wound site. In contrast, adult rabbit wounds showed increased expression of $TGF\beta_1$ and $TGF\beta_2$ protein by 7 days postwounding.[7] In a model of embryonic mouse limb healing, Martin et al demonstrated that TGFβ protein was detected within 1 hour postwounding, but disappeared by 18 hours postwounding.[8] Although TGFβ may be present in fetal skin wounds at levels that are below the threshold for detection using immunohistochemical techniques, these studies support the hypothesis that fetal wounds are relatively TGFβ-deficient compared with adult wounds.

TGFβ requires activation for biological activity. Immunohistochemical staining for $TGF\beta_1$ LAP detected this protein in adult wounds from 6 hours postwounding to 18 hours postwounding, but did not detect it in fetal wounds. Thus, inactive TGFβ is also not present in human fetal wounds.

A major difference between fetal and adult wounds is the degree of inflammation at the wound site and hence the cytokine profile of the wound. Previous studies have correlated the absence of scarring in fetal wounds with the sparse inflammatory response, as evidenced by reduced macrophage and monocyte infiltrates,[9] absence of endogenous immunoglobulins at the wound site,[10] and reduced angiogenesis. Macrophages are the principal source of TGFβ in adult wounds, and the reduced TGFβ level in fetal wounds may reflect the absent or minimal macrophage infiltrate in these wounds.[9] TGFβ is also released from the alpha-granules of activated platelets in adult wounds, and it is possible that fetal platelets do not synthesize or release TGFβ at the fetal wound site.

By adding $TGF\beta_1$ to 16-week-gestation human fetal skin, we promoted scarring in normally scarless wounds. Similarly, adding TGFβ to wound implants in fetal rabbits induced abnormal fibroblast penetration of the implants and a marked increase in collagen deposition.[3] TGFβ added to fetal human fibroblasts in culture increases collagen gene and protein expression.[11] This fibrotic response to TGFβ by fetal fibroblasts shows that these cells have TGFβ receptors as well as the cellular machinery to produce a scar.

Two important assumptions are inherent in our model of human wound healing. The first is that the human skin remains viable on the nude mouse flank. Fetal subcutaneous grafts have excellent viability and retain their native morphology when examined histologically. Second, the grafts are healing with human cells and human matrix, as the fetal skin would in its native location. Previous work validates this model by demonstrating that fetal subcutaneous grafts heal with matrix deposition by human fetal fibroblasts.[12]

This study suggests that anti-TGFβ strategies might ameliorate scar formation. Indeed, neutralizing antibodies to $TGF\beta_1$ or $TGF\beta_2$ markedly decreases scarring in adult rat wounds.[13,14] The neutralizing antibody-treated wounds have normal tensile strength and nearly normal dermal architecture compared with untreated wounds, and this effect is accompanied by deposition of less collagen and fibronectin, and infiltration by fewer macrophages and blood vessels. Application of TGFβ neutralizing antibody at the time of wounding (and not later) was essential to reduce active TGFβ levels, prevent autoinduction of TGFβ mRNA, and limit macrophage infiltration and further TGFβ release. Thus, TGFβ is inextricably linked to scar formation. TGFβ is absent in scarless fetal wounds; the addition of TGFβ to fetal wounds results in scar formation; the presence of TGFβ after adult injury correlates with the degree of fibrosis observed; and the blocking of TGFβ in adult wounds has a potent antiscarring effect.

Although injection of TGFβ neutralizing antibodies into wounds has limited clinical potential because of antigenicity problems, there are other promising ways to reduce wound levels of TGFβ. For example, decorin is a small proteoglycan that binds TGFβ, and systemic injections of decorin suppress the pathological increase in matrix synthesis in an animal model of glomerulonephritis.[15] Addition of the $TGF\beta_3$ isoform down-regulates $TGF\beta_1$ and β_2 levels in adult rat wounds and leads to a pronounced antiscarring result.[16] Application of the inexpensive and readily available sugar mannose-6-phosphate to rodent wounds also limits scar formation, presumably by blocking the insulinlike growth factor type II/mannose-6-phosphate receptor that is important for TGFβ activation. Other theoretical anti-TGFβ therapeutic strategies, such as flooding the wound with soluble TGFβ receptors to compete with cellular TGFβ binding sites or adding antisense oligonucleotides to inhibit TGFβ gene expression, may also make adult wounds heal in a fetallike scarless manner. Although these applications of wound pharmacology require clinical testing, anti-TGFβ therapeutic strategies may reduce scarring in children and adults.

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Discussion

T. Crombleholme (Boston, MA): I have several questions for Dr Sullivan. You point out in the manuscript that TGFβ isoform-3 has been shown in adult rat wounds to down-regulate $TGF\beta_1$ and $TGF\beta_2$, with antiscarring effects. Could this be playing a role in your model? Second, macrophages and platelets are a rich source of TGFβ. In the wounds that were exposed to exogenous TGFβ, was there any histological evidence of an inflammatory infiltrate?

Last, how do you interpret these findings, that is, the absent TGFβ, in view of previous work from your laboratory by Mike Longaker, demonstrating in fetal sheep that there were higher levels of $TGF\beta_2$ in fetal wound fluid as opposed to adult wound fluid? Are these species differences, or is the expression of cytokines compartmentalized in the wound?

K. Lally (Houston, TX): As Dr Sullivan mentioned, TGFβ is produced by a large number of cell types, in fact, most of the cell types in the body. The first question I have is, why do you think it is lacking in the fetal wound? What particular cell type is either not producing it or depressing production?

The second question relates to Dr Crombleholme's comment that TGFβ is a potent macrophage chemoattractant. Did you look at the relative amounts of macrophages in the wound? Did you stain specifically for macrophages in the wound?

Third, TGFβ may co-regulate with eicosanoids, specifically PGE_2. Have you had a chance to look at PGE_2 release in the fetal macrophage versus the adult?

E. Fonkalsrud (Los Angeles, CA): Could you tell us the difference in tensile strength between wounds that heal with scar and those without the collagen matrix in the scar?

D. Vane (Burlington, VT): Why do you suppose that the TGFβ you put into the disk was not picked up by the mouse circulation and distributed to the contralateral fetal wound?

K.M. Sullivan (response): $TGF\beta_3$ is a third isoform of TGFβ that has been shown to reduce scarring in adult animal models. Unlike my findings for $TGF\beta_1$ and $TGF\beta_2$, I found no difference in the amount of $TGF\beta_3$ in adult versus fetal wounds using immunohistochemistry; so $TGF\beta_3$ was not felt to play a major role in scarless fetal repair. However, it would be interesting to see whether a disk of $TGF\beta_3$ placed underneath an adult human wound would reduce scarring.

By immunohistochemistry, we have seen that there

is a markedly decreased inflammatory cell infiltrate in fetal wounds. Indeed, because inflammatory cells are a prime source of TGFβ, this may contribute to the lack of TGFβ at the fetal wounds.

We have performed in situ hybridization studies for TGFβ in these wounds. In situ hybridization is more sensitive than immunohistochemistry. Again, no TGFβ was found in fetal wounds, whereas TGFβ was detected in adult wounds.

We feel that placing a wire mesh cylinder under the skin of the sheep produces a "frustrated" wound environment—a wound that cannot heal. The cylinder's persistent irritation may alter healing, just as a foreign body does, and result in the high content of $TGF\beta_2$ in fetal sheep wound cylinder fluid.

Human and mouse macrophage staining have been performed in this model. We found no mouse macrophages and very few human macrophages in human fetal skin placed in the subcutaneous pockets on the flanks of nude mice.

Tensile strength measurements have been performed in our lab on fetal wounds in sheep. The fetal tissue is delicate, and there are technical difficulties that must be overcome.