Transforming Growth Factor $\beta_3$ Promotes Fascial Wound Healing in a New Animal Model

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Hypothesis: Transforming growth factor $\beta_3$ (TGF-$\beta_3$) promotes fascial wound healing in a new animal model, as measured by wound breaking strength, collagen deposition, and cellular proliferation.

Design/Intervention: Bilateral, longitudinal incisions were made in the anterior rectus sheaths of 24 male New Zealand white rabbits. One incision was treated with 1 µg of TGF-$\beta_3$; the contralateral incision served as a control. The wounds were harvested at 1, 2, 3, 4, 6, and 8 weeks after creation (“wounding”).

Main Outcome Measures: Wound tissue was tested for breaking strength using a tensiometer and processed for histological examination of collagen deposition and cellular proliferation at all time points after wounding. Collagen deposition and cellular proliferation were measured in histological cross sections of wounds with Masson trichrome staining and proliferating cell nuclear antigen immunohistochemistry, respectively.

Results: At all time points after wounding, treatment with TGF-$\beta_3$ significantly increased the wound breaking strength (up to 138%) and collagen deposition (up to 150%) over the control group. Cellular proliferation was increased during the first 3 weeks after wounding (up to 147%), but returned to baseline levels by the fourth week.

Conclusions: Transforming growth factor $\beta_3$ promotes fascial wound healing. In this new animal model of fascial wound healing, TGF-$\beta_3$ increased fascia breaking strength, collagen deposition, and cellular proliferation. These results are similar to findings in cutaneous wound models and demonstrate, for the first time, a pharmacologic agent to accelerate fascial healing.

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Surgeons have long recognized that the strength of abdominal wound closure relies on restoration of fascial integrity. Inadequate fascial healing can result in a number of potentially serious complications, including fascial dehiscence, incisional hernia formation, and even evisceration. Despite numerous research studies seeking to optimize outcomes, the incidence of wound disruption has changed little in the past 40 years. Studies examining mechanical factors in wound closure, technical variations in closure, incision orientation, suture material, and wound physiology have resulted in equivocal results with regard to the enhancement of healing strength and the reduction of dehiscence and herniation. Other than vitamin supplements for the nutritionally deficient, no pharmacological agent or technique has been shown to significantly improve fascial wound healing.

In the early 1950s, experimental evidence first demonstrated prolonged healing in fascia relative to skin and other tissues. In cutaneous wounds, inflammatory cells migrate from local blood vessels, and fibroblasts migrate from the wound margin to almost immediately influence healing. In contrast, inflammatory cells and fibroblasts must, to a greater extent, migrate through surrounding tissues to the wound area in healing fascia due to its limited cellularity and vascularity. Other studies demonstrated tissue-specific differences such as longer cell-doubling times, larger cell volume, and higher glucose requirements in fascial fibroblasts. As a result of these differences, skin requires 6 weeks to regain 60% of its unwounded strength, while similar percentage gains are not achieved in fascia until after 14 weeks.

The transforming growth factor betas (TGF-$\beta_1,2,3$ isoforms), considered to be the main orchestrators of wound healing, affect a broad range of activities in repair of injured tissue, including fascia. Transforming growth factor $\beta$ has direct effects on target cells and indirect effects...
MATERIALS AND METHODS

STUDY DESIGN

Twenty-four New Zealand white rabbits were wounded as described below. The wounds were treated with a single dose of 1-µg TGF-β3 in methylcellulose gel or vehicle alone at the time of wounding. This dose has been previously proven optimal in incisional and excisional wounds of similar size.16 The fascial incisions were harvested at 1, 2, 3, 4, 6, and 8 weeks after wounding; 4 animals were used at each time point. Weeks 5 and 7 were not tested to conserve resources. Each incision was analyzed for breaking strength, collagen deposition, and cellular proliferation, and the results were averaged for each animal. Animals were housed under standard conditions and fed ad libitum under an experimental protocol approved by the Northwestern University Animal Care and Use Committee.

All wounds were created and harvested in a matched fashion, and the data collected in a manner to allow paired analysis, with each animal serving as its own control. A paired, 2-tailed t test was performed at each time point for wound breaking strength, collagen deposition, and cellular proliferation. P<.05 was considered significant. Statistical analysis was performed using SPSS 8.0 software for Windows (SPSS Inc, Chicago IL).

ANIMAL MODEL

Adult male rabbits, weighing 2 to 2.5 kg, were anesthetized with intramuscular injection of 60-mg/kg ketamine hydrochloride and 5-mg/kg xylazine hydrochloride, and prophylactically treated with 1 million units of intramuscular penicillin G prior to making the skin incision. The abdominal area was shaved and prepared with povidone-iodine solution. A sterile technique, a longitudinal, midline skin incision was made from the xiphoid process to 2 cm above the pubic tubercle, down to the level of the linea alba. Soft tissues were dissected from the anterior aspect of both rectus sheaths, in the avascular prefascial plane. Bilateral, longitudinal, 6-cm incisions were made in each rectus sheath under surgical loupe magnification, taking care to not injure the underlying muscle (Figure 1). The incisions were randomized to receive either 1 µg of TGF-β3 isoform in methylcellulose vehicle or vehicle alone. Vehicle was previously compared with nontreated control, and no difference in healing was found (unpublished data). The fascial incisions were closed with a running 6-0 nylon suture, and the skin was closed with a 4-0 nylon suture.

WOUND TENSILE TESTING

At 1, 2, 3, 4, 6, and 8 weeks after wounding, the animals were humanely killed and the anterior rectus fascia harvested. After removing the nylon fascial suture, a template with parallel surgical blades was used to excise 5-mm strips perpendicular to the incision. Eight strips were taken from each wound; 6 strips were used for breaking strength testing, and 2 for histological analysis. The maximum load tolerated by wound strips prior to breaking was measured in newtons with a tensiometer (Instron Inc, Canton, Mass). The 2 remaining strips were formalin fixed and embedded in paraffin for histological evaluation.

COLLAGEN DEPOSITION

Histological slides were prepared with Masson trichrome staining for evaluation of collagen deposition (Figure 2). Because of the sensitivity of trichrome staining to errors in technique and to reduce variations in staining between specimens, all tissue was prepared and stained together in mass. Slides were viewed under a standard light microscope at ×20 power. Using a grading scale of 0 to 4, two blinded observers (J.W.T. and J.R.M.) graded the collagen deposition of treated and untreated specimens. In addition, computerized histological analysis was performed using a software program (NIH Image; National Institutes of Health, Bethesda, Md) to obtain semiquantitative results. Briefly, digital images were captured using a microscope (Nikon Axioscope; Nikon Inc, Melville, NY) equipped with a color digital camera. These images were imported into a personal computer running the NIH Image software. This software program allows quantification of specific histological tissues by measurement of individual pixels, based on intensity and color, allowing the user to select a specific stain and determine the relative area of a slide that the stain occupies.

IMMUNOHISTOCHEMISTRY: PROLIFERATIVE CELLULAR FRACTION

Histological slides were prepared by immunohistochemical staining for proliferating cell nuclear antigen (PCNA) to determine cellular proliferation, and counted by 2 blinded observers (Figure 3). Briefly, proliferating cells were identified using a monoclonal antibody to the PCNA (Calbiochem-Novabiochem, San Diego, Calif). This 37-kd protein has been used as a reliable marker for the determination of proliferative cellular fractions in hematological and solid malignant neoplasms as well as benign tumors.27 In accord with our previous dilution curve development, the primary antibody (1:100 dilution) was applied to the slides and incubated at room temperature overnight. Rabbit-absorbed, biotinylated secondary antibody was applied (Super Sensitive Detection System; Biogenix, San Ramon, Calif) and incubated for 20 minutes at 37°C. Streptavidin-horseradish peroxidase complex was applied and incubated at 37°C for 20 minutes. 3-Amino-9-ethylcarbazole (AEC) chromogen labeled the nuclei of proliferating cells in red. Slides were counterstained and mounted with aqueous medium in standard fashion. The specimens were analyzed by 2 blinded observers and reported as nuclear counts per high-power field. All spindle-shaped (representing fibroblasts) and round nuclei (representing inflammatory cells) up to 5 mm on each side of the incision were counted. The final results were expressed as percentage change vs controls.

(on the production and release of other growth factors involved in wound healing.16-19 In vivo and in vitro studies have shown that TGF-β is a potent stimulator of procollagen alpha 1[1] production by fibroblasts.20-22 Of the 3 mammalian isoforms, the TGF-β3 isoform is the most potent with regard to these characteristics.23 Based on our previous work with TGF-β3 isoform...
we hypothesize that homologous stimuli drive wound healing in both skin and fascia, and that TGF-β}_3 will accelerate fascial healing.

It is the purpose of this work to examine the role of growth factor–stimulated healing in a fascial model. We introduce a modification of Lichtenstein and coworkers’ rabbit model for fascial healing, which we believe is an ideal model for the study of recombinant growth factors in fascial healing.

The outcome measures investigated in this study included wound breaking strength, collagen deposition, and cellular proliferation in response to TGF-β}_3 stimulation of wound healing.

### RESULTS

#### TENSILE TESTING

The average breaking strength of 6 test samples was compared with that of 6 control samples from the same animal, 4 animals per time point (Table). A statistically significant increase in wound breaking strength was seen with TGF-β}_3 treatment as early as 1 week after wounding and continued throughout the study. The wound breaking strength of the TGF-β}_3–treated specimens was augmented 22% at week 1, peaked at 39% 2 weeks after wounding, and stabilized at 27% increase of control by 6 weeks (P<.01) (Figure 4A). At the latest time point, 8 weeks after wounding, the increase in breaking strength of the test group continued to demonstrate a 27% increase over the control group.

#### COLLAGEN CONTENT

Collagen content as evaluated by blinded observers and computer-aided histological analysis closely paralleled the breaking strength results (Figure 4B). Blinded observation demonstrated a 25% increase in collagen deposition with TGF-β}_3 treatment at week 1, peaking at 50% increase during week 3, and stabilizing with a 25% increase over the control specimens. Computer-aided histological analysis demonstrated a similar increase with TGF-β}_3 treatment over the control condition (P<.05). At each time point, the observational results correlated closely with the computer analysis.

### CELLULAR PROLIFERATION

Cellular proliferation as determined by PCNA staining was significantly increased for the first 3 weeks of testing (P<.05). Proliferation was increased in both spindle-shaped cells (representing fibroblasts) and round cells (representing all other cells) equally. No significant difference was seen in proliferation between cell types. At 1 week after wounding, total PCNA counts were increased over control (+47%). A 21% and 9% gain in cellular proliferation was seen in treated specimens vs control specimens at 2 and 3 weeks, respectively. At 4 weeks, the PCNA counts returned to baseline, with no statistical difference from control (Figure 5).
wounded tissue to heal stronger than normal wound healing. In contrast to previous studies that have shown that wounds regain maximally only 80% of the breaking strength of unwounded tissue, tensile testing performed on unwounded tissue displayed a breaking strength similar to (22-25 N) that is required to break the TGF-β3-treated specimens at 8 weeks after wounding (approximately 90% of unwounded strength for TGF-β3-treated vs 65% for control). These results suggest that modulation with growth factors can assist a healing tissue in attaining closer to 100% of the prewound strength.

**Figure 2.** Histological analysis of control (A, C, and E) and transforming growth factor β3–treated (B, D, and F) wounds at 2, 4, and 8 weeks after wounding. The unwounded margins of the native fascia (NF) are seen laterally as a homogeneous blue band, whereas the media fascial incisions (IN) are more heterogeneous in appearance. Collagen is stained intensely blue (trichrome stain, original magnification ×5).

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Collagen deposition as evaluated by computerized image analysis was effective for estimating the amount of collagen present in histological specimens. The amount of collagen present as determined by computerized histological analysis closely paralleled the blinded observer results and correlated closely with the breaking strength. Only during the first 3 weeks after wounding did the collagen deposition mildly overestimate the breaking strength results. We believe this is likely due to poor cross-linking of collagen fibers in the early stages of wound healing, resulting in excess collagen with little tensile strength.

The results of the cellular proliferation by PCNA counts demonstrated that initial cellular proliferation is enhanced. Although no difference in proliferation was seen between spindle-shaped cells and round cells, the increase in total cellular proliferation was highly significant. As expected from a single dose of growth factor, the increase in proliferation was temporary, returning to basal levels by 4 weeks after wounding. Use of a delayed-release formulation may be of benefit in the clinical setting by prolonging the stimulatory effect, and further increasing the cellular proliferation and total collagen deposition.

The ability to augment fascial healing with growth factors has clinical potential in high-risk abdominal wall closures and other fascial defects, as well as routine herniorrhaphy. As early as 1 week after wounding, TGF-β3 treatment significantly increased the wound breaking strength. This rapid gain in strength may help augment an incision at a time when the tissues are at their weakest and the suture is degrading. The sustained increase in collagen and breaking strength offers some evidence that TGF-β3 may also be able to counter the effects of poor tissue integrity, atrophy due to advanced age, or various disease states. Although no complications were noted in any of the animals, potential drawbacks of using TGF-β3 to augment fascial wound healing in humans may include development of intra-abdominal adhesion or increased scar formation. While not yet addressed in human subjects, postoperative peritoneal adhesion formation in animals treated with intra-abdominal injection of TGF-β has been minimal. In contrast to the other TGF-β isoforms, treatment with TGF-β3 exhibits no increased scar formation, and is therefore the most promising isoform for clinical use.

Another interesting use of growth factor therapy may be in combination with mesh. Synthetic materials are frequently used to reinforce hernia repairs, and the stimulatory effects of TGF-β3 could accelerate tissue incorporation of these materials. This may reduce the incidence of recurrent hernia formation due to dislodgment of the mesh during the early postoperative period, such as in laparoscopic herniorrhaphy, where the mesh is usually

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Figure 3. Histological specimens stained for proliferating cells in control (A) and transforming growth factor β3–treated (B) wounds at 1 week after wounding. Proliferating cells are stained red-orange (proliferating cell nuclear antigen stain, original magnification ×100).
held in place by just a few stay sutures or staples. In addition, the increase in breaking strength seen with TGF-β3 in healing fascia may also allow the surgeon to choose absorbable mesh in instances where one otherwise might use permanent, nonabsorbable mesh, or even allow the surgeon to perform repairs without the use of mesh in cases that one might otherwise use it. Further work is needed to determine the most effective method of delivery, whether alone, with fascia or dermal allograft, or in combination with mesh, and clinical studies examining these points are warranted.

The TGF-β3 was supplied by David Cox, PhD, Novartis Pharma AG, Basel, Switzerland.

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REFERENCES