Early Growth Response Factor-1: Expression in a Rabbit Flexor Tendon Scar Model

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Reduction of postoperative adhesion formation after flexor tendon repair has been a focus of experimental research for decades. Modification of the wound healing environment has not resulted in the reduction of postrepair adhesions. Early postoperative motion protocols have been developed to improve flexor tendon excursion after repair, but hand surgeons continue to search for improved means of reducing adhesion scar formation.

Tendon excursion requires unobstructed passage through the fibro-osseous tunnel for maintenance of normal range of motion at the interphalangeal and metacarpophalangeal joints. Peritendinous scar formation represents the prime culprit of postoperative morbidity in tendon laceration repairs.

Prevention of the adhesion formation process has evolved from mechanical and biochemical interventions to a current focus on molecular biology.1 Multiple peptide growth factors have been identified as potential inhibitors of adhesion formation, and EGR-1, a transcription factor associated with the synthesis of a variety of key fibrotic growth factors and expression of extracellular matrix genes, has never been identified in a tendon repair model.

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Received for publication May 6, 2011; accepted September 14, 2011.
Copyright ©2012 by the American Society of Plastic Surgeons
DOI: 10.1097/PRS.0b013e3182402d81

Disclosure: The authors have no financial interest in any of the products or devices mentioned in this article.

This work was supported by THE PLASTIC SURGERY FOUNDATION.
identified in the flexor tendon healing process, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor, transforming growth factor-β (TGF-β), epithelial growth factor, and basic fibroblast growth factor in intrinsic tendon cells, surrounding sheaths, and infiltrating inflammatory cells.2–8

The role of TGF-β in flexor tendon healing and pathogenesis of scar is well documented.9–15 In vitro attempts at inhibition of TGF-β within its intracellular signaling pathway have not fully suppressed collagen production.16 This is not considered a fault of the anti–TGF-β strategy, as suppression of a single molecule would be unlikely to fully achieve such an effect. Focusing on biomechanical testing of the anti–TGF-β effect, in vivo work on extracellular neutralization of TGF-β demonstrates improved tendon motion after repair.13 Katzel and colleagues recently demonstrated improved range of motion (after tendon repair) in Smad3−/− mice, compared with controls, in their investigation on the effects of abrogating the canonical TGF-β intracellular Smad signaling pathway.17

Intracellular/intranuclear control of TGF-β downstream signaling provides an alternative focus for adhesion formation control. We do not currently consider this focus more effective than well-established techniques of TGF-β ligand-receptor inhibition; instead, we believe the concept is novel and worthy of scientific investigation.

Our laboratory previously studied EGR-1 and its up-regulation in the proinflammatory setting of ischemia-reperfusion injury.18 There is accumulating evidence pointing to the important role of EGR-1 in fibrosis.19 EGR-1 is part of a novel and apparently Smad-independent intracellular TGF-β signaling pathway that is required, along with Smad, for mediating full stimulation of the collagen gene.19

EGR-1 is a short-lived, immediate early response gene located on chromosome 5q31.20–22 It was first identified as an “early growth response gene” by Sukhatme et al. in 1988.23 Despite its negligible detection in normal cells, it is rapidly expressed in response to extracellular stimuli (i.e., mechanical injury, shear stress, growth factors, cytokines).19,21,22,23 The EGR-1 gene links external stimuli with multiple cellular signaling pathways by altering the expression of its product, EGR-1 transcription factor, with resultant enhanced expression of its transcription factor’s target genes (i.e., VEGF, PDGF, FGF2, fibronecctin, MMP, TGF-β1, intercellular adhesion molecule-1, tissue factor, tumor necrosis factor-α).21,24,25 Because EGR-1 preferentially binds to a DNA sequence found in the promoter region of many target genes, it essentially functions as a transcriptional master regulator.19 Targeting EGR-1 potentially represents another approach to minimization of fibrotic and inflammatory processes that plague flexor tendon repairs. No study has sought to identify the presence of EGR-1 in a healing tendon wound bed. Furthermore, and to the best of our knowledge, TGF-β and EGR-1 have not been concurrently inhibited within a tendon repair model to compare their respective effects on postoperative tendon range of motion.

The purpose of our study was two-fold. The primary aim was to determine whether EGR-1 is expressed at the site of a healing flexor tendon repair. Once identified, future studies could attempt to inhibit EGR-1 expression and evaluate for effect on range of motion, TGF-β expression, and tendon tensile strength after flexor tendon repair. We also aimed to develop a reproducible, standardized model of scar formation in the healing flexor tendon. A standard model, through which various molecular targets are experimentally manipulated, could potentially provide a more objective, quantifiable means of assessing tendon adhesion scar formation in future studies. Various products were considered during our sclerosing agent selection process. We desired an agent that had been shown to enhance collagen formation in other basic science studies, was liquid based, and was easy to apply to the repair model we used. Sodium morrhuate, a liquid agent used in venous sclerotherapy, was identified as our experimental agent of choice. It has been previously implicated in collagen fibril thickening.26 We hypothesized that sodium morrhuate–treated tendons would facilitate greater stimulation of fibroblastic collagen production than control saline-treated tendons, and that EGR-1 would be found in the healing tendon wound bed.

**MATERIALS AND METHODS**

**Tendon Repair**

A standard, reproducible model of flexor tendon repair was used for the experiment.27 Thirty adult male New Zealand White rabbits (4.0 to 4.5 kg) were anesthetized with intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). Supplementary anesthesia was provided with isoflurane 1 to 3%. Ceftiour sodium (Pfizer, Inc., New York, N.Y.) (2 mg/kg) was administered immediately preoperatively for antibacterial prophylaxis. Experimental forepaws underwent isolation of the middle digit flexor digitorum profundus equivalent, with sharp transection in zone II, and immediate repair using the modified Kessler tech-
nique with 6-0 nonabsorbable monofilament suture. In the experimental group ($n = 3$ rabbits per time point), 0.05 ml of sodium morrhuate (American Regent, Inc., Shirley, N.Y.), a sclerosing agent, was dripped atop the repair site after partial closure of the sheath with 6-0 nonabsorbable monofilament suture to contain the product locally. Sheath closure was then fully completed. In the operated control group ($n = 3$ rabbits per time point), 0.05 ml of phosphate-buffered saline was dripped atop the repair site in similar fashion. To offload the tendon at the repair site, the flexor digitorum profundus tendon was divided 1.5 cm proximal to the repair and sutured loosely to the flexor digitorum superficialis equivalent using a single 6-0 nonabsorbable monofilament horizontal mattress suture. This technique eliminates the need for postoperative forepaw casting (Thorfinn J, Chang J, personal communication, March 23, 2009). Skin was approximated with 4-0 nonabsorbable monofilament suture. Wounds were dressed with bacitracin and Telfa (Tyco Healthcare, Inc., Mansfield, Mass.). Forepaws were wrapped with Webril (Tyco Healthcare), Conform stretch bandage (Tyco Healthcare), Coban (3M, St. Paul, Minn.), and Tensoplast (BSN Medical, Inc., Charlotte, N.C.). Animals were killed with intravenous SleepAway (Fort Dodge Animal Health, Fort Dodge, Iowa) (2 ml/4.5 kg) at 1, 3, 7, 14, and 28 days postoperatively. Operated experimental and control tendons and negative control, unoperated, contralateral forepaw tendons were harvested.

### Tendon Harvest

Original cutaneous incisions in the operated forepaws were reopened sharply. Tendons were dissected meticulously to ensure removal of both the repair site and sheath en bloc. The contralateral, unoperated control forepaw was then opened and removed carefully to include both tendon and sheath en bloc. Specimens were stored in 5% formaldehyde overnight and paraffin embedded. Six-micron slices were subjected to routine Masson trichrome staining and immunohistochemistry.

### Immunohistochemistry

Immunohistochemistry was performed using anti–EGR-1 antibody (S-25; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), biotinylated secondary antibody, and avidin-horseradish peroxidase complex (ABC kit; Vector Laboratories, Burlingame, Calif.). Detection was performed with DAB (Vector Laboratories). Slides were counterstained with hematoxylin and coverslipped.

### Histologic Analysis

#### Scoring Tissue Sections

Representative experimental (sodium morrhuate), operated control (phosphate-buffered saline), and unoperated control slides from each postoperative harvest time point were evaluated by two independent reviewers.

For analysis of collagen scar production, Masson trichrome–stained slides of the tendon repair sites subjected to sodium morrhuate or phosphate-buffered saline were analyzed under 40× magnification with an Olympus BX50 digital light microscope (Olympus America, Inc., Center Valley, Pa.). Slide images were captured with Spot Basic Imaging software (Diagnostic Instruments, Sterling Heights, Mich.). Images were then divided into 15 equivalent rectangles ($16 \times 20 \mu m$ each), as measured with Scion Image for Windows software (Scion Corp., Frederick, Md.). The extent of blue “scar staining” ($n$) was judged independently by each reviewer, and a ratio was calculated ($n/15$) for each slide. Respective tendon “scar ratio” scores (from each reviewer) were averaged together, and a final scar ratio score was generated for each slide. These final scar ratio means, comparing sodium morrhuate to phosphate-buffered saline tendons, were statistically analyzed for tendons harvested at 1 week postoperatively. One week was chosen as the time point for analysis, as it corresponds with a phase of active and rapid collagen deposition in the proliferative stage of wound healing. Representative slides are shown in Figure 1.

For immunohistochemistry, representative images of experimental (sodium morrhuate), operated control (phosphate-buffered saline), and unoperated control sections were analyzed under 100× magnification with an Olympus BX50 light microscope (Olympus America). Representative slides are shown in Figure 2. The images in Figure 3 are 400× magnification of the same images in Figure 2. Slide images were captured with Spot Basic Imaging software (Diagnostic Instruments). The intensity of EGR-1 staining was graded independently, by each reviewer, on a scale of 1 (no staining) to 5 (strong staining) for each slide. Respective tendon EGR-1 staining intensity scores (from each reviewer) were averaged together, and a final mean score was generated for each slide. The final mean staining intensity scores for tendons exposed to sodium morrhuate and phos-
phate-buffered saline were compared across all time points.

**Statistical Analysis**

Differences between collagen scar ratio means for tendons exposed to sodium morrhuate and phosphate-buffered saline were analyzed with the unpaired *t* test. Significance was established at *p* < 0.05. EGR-1 staining intensity grade differences between sodium morrhuate and phosphate-buffered saline were analyzed across all time points using the unpaired *t* test. Significance was established at *p* < 0.05.

**RESULTS**

**Creating a Scar Model**

Mean ratios of collagen staining were compared between tendons subjected to sodium morrhuate and phosphate-buffered saline at 1 week postoperatively: 0.7783 ± 0.03 and 0.7667 ± 0.02, respectively. No statistically significant difference in collagen staining ratios was identified (*p* = 0.76). Sodium morrhuate tendon treatment groups did not generate more scar than phosphate-buffered saline tendon control groups in our model.

**EGR-1 Expression**

Sodium morrhuate and phosphate-buffered saline groups were compared to identify potential effects of either agent. No group difference was demonstrated when comparing phosphate-buffered saline to sodium morrhuate for effect on EGR-1 expression intensity. After averaging mean group intensity data scores, the two tendon groups (sodium morrhuate and phosphate-buffered saline) were examined collectively across postoperative time points. EGR-1 expression followed a bell-shaped curve of expression over time in the flexor tendon repair model, with staining intensity peaking at postoperative day 7. A statistically significant difference was demonstrated when comparing EGR-1 staining intensity scores from postoperative days 3, 7, and 14 to day 28 (Fig. 4). We demonstrated, for the first time, that EGR-1 is expressed in a healing flexor tendon model.

**DISCUSSION**

Identification of the various growth factors involved in the normal process of flexor tendon healing, and the pathologic state of tendon adhesion scar formation, has been elucidated over the previous two to three decades. Despite these findings, application has yet to move from bench to bedside. Early motion protocols, advocated by Kleinert, Duran, and Strickland, are the only means of attempting to limit the effects of adhesion formation currently available. Even with early motion rehabilitation, obtaining excellent results for active digital joint motion after primary flexor tendon repair remains a formidable challenge.

![Fig. 1. (Left) Collagen staining of a sodium morrhuate-subjected tendon repair site 1 week postoperatively (original magnification, ×40). (Right) Collagen staining of phosphate-buffered saline-subjected tendon repair site 1 week postoperatively (original magnification, ×40).](image-url)
Having a reproducible scar model for the field of hand surgery would potentially prove useful to investigators focused on manipulation of adhesion formation after tendon repair. Objective, quantifiable changes to a tendon adhesion model, with experimental manipulation, may make a study’s results more robust. The tendon adhesion model we hoped to generate aimed to enhance adhesion formation by application of sodium morrhuate, a substance known to contribute to collagen fibril diameter thickening. This attempt proved ineffective, as a statistically significant difference in collagen staining between tendons exposed to sodium morrhuate and phosphate-buffered saline was not found. We will acknowledge the associated limitations of this component of our investigation shortly.

Researchers have identified TGF-β as the preeminent factor in pathologic scar processes. Its neutralization in a dermal wounding model resulted in reduced inflammation and extracellular matrix deposition, with maintenance of tensile strength and normal dermal architecture. Our literature has demonstrated improvement in postoperative passive range of motion in a rabbit flexor tendon repair model after TGF-β1 neutralization, and inhibition of downstream signaling after TGF-β ligand-receptor interaction. Intracellular/intranuclear control of TGF-β downstream signaling provides an alternative focus for adhesion formation control.

There is accumulating evidence pointing to the important role of EGR-1 in fibrosis. Elevated and persistent EGR-1 expression has been detected in synovial fibroblasts in the collagen-rich subsynovial space of patients with rheumatoid arthritis, in atherosclerotic plaques, in fibrotic kidneys in rats with ureteral obstruction, in a model of peri-
tional adhesions, in pulmonary artery fibroblasts in hypoxic animals, in lung tissue from patients with emphysema, in chronic inflammation of inflammatory bowel disease, and in fibrotic skin and lung tissue from patients with scleroderma.  

EGR-1 stimulates TGF-β synthesis in a variety of mesenchymal cell types, enhances their sensitivity to TGF-β, and mediates the stimulation of collagen gene transcription elicited by TGF-β.  

Furthermore, in vitro studies have demonstrated a rapid and dose-dependent increase in EGR-1 protein in response to TGF-β stimulation of normal fibroblasts.  

EGR-1 also mediates the synthesis of key mediators (PDGF, basic fibroblast growth factor, thrombospondin, TGF-β, type 2 TGF-β receptor, tumor necrosis factor-α, intercellular adhesion molecule-1) in the fibrotic/inflammatory response, and contributes to the transcription of several extracellular matrix genes that exacerbate fibrosis (collagen, fibronectin, and tissue inhibitor of metalloproteinase-1).

Multiple subspecialties in the medical field have begun to elucidate the role of EGR-1 in the management of the fibrotic diseases they treat. EGR-1 has been identified as a convergence point for many signaling cascades. Experimentally induced attenuation of EGR-1’s effect on scar formation has yielded therapeutic improvements in a variety of experimental models of cardiovascular, pulmonary, dermatologic, immunologic, and hepatic disease. Considered together, these results have led us to hypothesize that EGR-1 inhibition may lead to reduced fibrosis in a flexor tendon repair model.

Specific molecular suppressors of EGR-1 include antisense oligonucleotides, decoys, small interfering RNA, DNAzymes, and NGFI-A binding proteins. NGFI-A binding proteins 1 and 2 are transcription cofactors that bind to the inhibitory domain of the EGR-1 gene, serving to block its biological activity. NGFI-A binding protein 2 expression is, itself, enhanced by EGR-1, essentially allowing EGR-1 to impose its own system of self-regulated expression. Furthermore, EGR-1 can bind to the EGR-1–binding sequence of its own gene, resulting in down-regulation of its own transcription.

Targeting the EGR-1 gene with specific DNAzymes in a rat model of ureteral obstruction resulted in reduced fibrosis and myofibroblast marker expression. Several medications in clinical use have potent inhibitory effects on EGR-1 expression or activity. These include mycophenolate mofetil (CellCept; Genentech, South San Francisco, Calif.), cyclosporine, simvastatin (Zocor; Merck & Co., Inc., Whitehouse Station, N.J.), rosiglitazone, and the tyrosine kinase inhibitor antineoplastic drug used in hematologic malignancies, imatinib mesylate (Gleevec; Novartis, East Hanover, N.J.). Administration of simvastatin to mice with advanced atherosclerotic lesions resulted in reduced lesional levels of tissue factor, fewer inflammatory macrophages, and reduced expression of EGR-1. In vitro analysis of macrophages from these same mice demonstrated reduced binding of nuclear proteins to EGR-1–binding sequences following pretreatment with simvastatin.

We acknowledge the limitations of our study, specifically within the context of attempting to create a scar model. The chosen postinjury time point for comparing histologic scar production...
between sodium morrhuate and phosphate-buffered saline was suboptimal. One week after injury is early in the proliferative stage of wound healing, with formal, mature, and organized scar not available for analysis. Repeating our study with comparison of scar tissue formation, between control and experimental arms, at 4 and 8 weeks may generate a more useful analysis. In hindsight, histologic analysis of two processes that may be temporally unrelated (mature scar formation and EGR-1 expression), using tendons procured at the same time point for each process, was suboptimal. The rationale behind tendon harvest time point selection supported the primary aim of this study—to identify EGR-1 in the healing tendon wound bed. EGR-1 is a short-lived, immediate early response gene. Time points were chosen to capture the early phases of wound healing that corresponded to when we suspected EGR-1 transcription factor expression to be greatest. TGF-β has been shown to induce rapid and transient accumulation of EGR-1 protein and mRNA in normal human skin fibroblasts, peaking at 30 minutes and persisting for 120 minutes.19

Our technique for quantifying EGR-1 expression also had limitations. Future studies may not replicate our temporal expression pattern if more formal quantification methods are used (i.e., Western blot analysis and polymerase chain reaction). However, our goal of identifying EGR-1 in a healing tendon wound bed, with immunohistochemistry, was accomplished.

We recognize that the significance of this finding is not yet established and also acknowledge that the time point of peak EGR-1 expression after repair does not coincide with the timeframe of mature scar development (i.e., weeks 4 to 8 after injury). However, as stated, several other organ system scar models note therapeutic improvement in injury). However, as stated, several other organ system scar models note therapeutic improvement (i.e., weeks 4 to 8 after repair does not coincide with the timeframe of mature scar development (i.e., weeks 4 to 8 after injury). However, as stated, several other organ system scar models note therapeutic improvement (i.e., weeks 4 to 8 after repair). Forthcoming studies will focus on EGR-1 inhibition with one of the agents previously noted, administered postoperatively to rabbits undergoing flexor tendon repair. Subsequent data analysis will include assessment of levels of EGR-1 and TGF-β expression, tendon excursion, and tendon tensile strength. Future studies may also consider a comparison of tendon excursion and tensile strength after TGF-β versus EGR-1 inhibition.

CONCLUSIONS

The ideal tendon scar model with which to test various products’ effects on adhesion formation remains undetermined. More refined quantification, and appropriate time point selection for scar analysis, may serve future studies that use sodium morrhuate in generation of a tendon scar model. We now know that EGR-1 is present in a healing flexor tendon wound bed. Future study will be directed toward manipulating EGR-1 expression in the established model of flexor tendon laceration repair, followed by determination of the resultant effects this manipulation has on the balance of adequate tendon tensile healing and the prevention of adhesions/tendon excursion after injury.

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ACKNOWLEDGMENTS

This work was supported by a Southern Illinois University School of Medicine, Department of Surgery Research Grant in 2008 and by a Plastic Surgery Foundation Pilot Research Grant in 2009. The authors thank Steven J. Verhulst, Ph.D., from the Department of Statistics and Research Consulting at Southern Illinois University School of Medicine, for contributing to the statistical processing of their data.

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