Published in final edited form as:

Plast Reconstr Surg. 2012 February ; 129(2): 300e–306e. doi:10.1097/PRS.0b013e31823aeb5a.

Evidence Translocation of Collagen Fibril Segments Plays a Role in Early Intrinsic Tendon Repair

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Abstract

Background—Severed tendon repair advances either with a scar through extrinsic repair or with regeneration through intrinsic repair. The hypothesis does intrinsic tendon repair reintroduce embryonic fibrillogenesis, whereby preformed, collagen “Fibril Segments” (FS) are incorporated into growing collagen fibers at wound edges?

Methods—Isolated tendons from 10-day-old chicken embryos were suspended in 1mg/ml of gentamicin antibiotic for 90 days, which released FS that were fluorescently tagged with rhodamine. Tendons Isolated from 14-day-old chicken embryos were wounded to half their diameter and then maintained as explants in stationary organ culture. Fluorescent-tagged FS (FTFS) were introduced to wounded tendon explants in the presence of high concentrations of neomycin, an antibiotic; cyclohexamide, a protein synthesis inhibitor; cytochalasin D, a disruptor of microfilaments, and colchicine, a disruptor of microtubules. At 24 hours explants were viewed with a fluorescent microscopy.

Results—Untreated, wounded tendon explants showed the translocation of FTFS from the explants’ surface to accumulation at wound edges. In the presence of high concentrations of neomycin, cytochalasin D or colchicine; FTFS failed to accumulate at wound edges and were retained on the explant surface. Inhibition of protein synthesis by cyclohexamide did not alter the accumulation of FTFS at wound edges.

Conclusion—Inhibiting FTFS accumulation by antibiotics is consistent with their role in the releasing of fibril segments. Experimental findings show fibril segments translocation and accumulation at wound edges involves microfilaments and microtubules, but not protein synthesis. The experiments support the hypothesis that intrinsic tendon repair advances through the incorporation of FS at wound edges.

INTRODUCTION

Our understanding of embryonic tendon fibrillogenesis has progressed further¹ than our understanding of tendon repair.² Tendons are composed of thick, robust, mostly type I collagen fibers.³ The basic collagen unit, traditionally called tropocollagen, consists of three 1,000 amino acids polypeptide chains, wrapped in a tight triple helix. Type I tropocollagen has 2 α 1(I) and 1 α 2(I) polypeptide chains. The α -chains are stabilized in a triple helix through the hydrogen bonding of hydroxyproline and the occurrence of glycine at every

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The work was presented at the Plastic Surgery Research Council Meeting in San Francisco, CA in 2010.

Funding Disclosure and Products

The work was supported by NIH grant #GM056851. The authors declare that there are no conflicts of interest or any commercial associations to disclose.

third residue. Tropocollagen is a rigid rod, 300 nm in length and 1.5 nm in diameter. Non-helical sequences, called telopeptides, are located on both the C and N terminal ends of tropocollagen. These peptides are important in generating and stabilizing the ordered packing of tropocollagen into collagen fibrils. Collagen fibers orientation within tendon fascicles is critical for tendon function. Tendon collagen fibers mostly run in longitudinal arrays, parallel to the direction of force. There are minor populations of identified collagen fibers that run in spirals, have horizontal or transverse orientations, but parallel orientation is the major orientation of collagen tendon fibers.⁴ The grouping of collagen fibers or bundles within tendon fascicles constitutes the tendon fiber. Along with the collagen fibers, tendon fibroblasts, called tenocytes, reside within tendon fascicles. Tendon fascicles are grouped together within a tendon sheath, where epitenon cells populate the outer shell and endotenon cells reside between the tendon fascicles.⁵ Tendons transfer forces of linear tension from muscle to bone. The flexor tendons of the hand transmit gross, high-magnitude forces for actions such as grasping. The ability of the tendon to relay force and slide reliably in its sheath results from its biological design.

Scarring, as consequence of tendon injury, disrupts the functional capacities of a tendon by weakening it. Optimizing regenerative healing, referred to as intrinsic tendon repair, terminates in the restoration of near normal tendon morphology and strength. The working hypothesis is intrinsic tendon repair results from the reestablishment of embryonic tendon fibrillogenesis. During tendon development, collagen fibril segments (FS) are the intermediate structural unit between tropocollagen and the assembly of tendon collagen fibers.^{1, 6} Intrinsic tendon repair is the reestablishment of fibrillogenesis.⁷ Here an organ culture model studies isolated wounded chicken embryo tendon explants that are maintained on a filter membrane within a specialized organ culture dish with serum supplemented culture medium. The accumulation of fluorescent tagged fibril segments (FTFS) at wounded edges of tendon explants by their physical translocation is followed over a 24 hour period. Cell biology processes involved in the movement of FTFS, their translocation, and their accumulation at wound edges are the central foci of the study.

METHODS

Fertilized chicken eggs, from the GemWillow Farm (Grantville, PA) were incubated in a poultry incubator. Tendons were isolated by pulling on all the toes from bilateral lower extremities of 10-day-old chick embryos and then they were suspended in 5 ml of phosphate buffered saline with gentamicin at 1mg/mL (Sigma-Aldrich Co., St. Louis, MO). The tendon- antibiotic suspension was maintained at 4 °C in a sealed 12 ml centrifuge tube for 90 days with constant agitation. The suspended tendons were centrifuged at $1,000 \times g$ for 1 min and the supernatant fraction, containing the FS, was saved.

Transmission electron microscopy (TEM) was used to confirm the identity of FS.¹¹ FS were fluorescently tagged by suspending FS in 10 mM Tris buffer at pH 7.0 on ice. About 10 mg of Lissamine rhodamine (Rh) B-200 on celite (USB Corp., Cleveland, OH) was added and the mixture vortexed, then immediately centrifuged and the supernatant saved, while the pellet containing celite was discarded. This method covalently bounded a limited number of rhodamine molecules to FS, now referred to as fluorescent tagged fibril segments (FTFS). Limiting the amount of rhodamine bound to FS by not optimizing pH, temperature, and reduced time for the coupling reaction to proceed, reduced the possibility of steric hindrance interfering with FTFS associating with growing collagen fibers. Leg tendons harvested from 14-day-old chick embryos were placed in Dulbecco's modification of Eagles medium (DMEM) with 10% new born bovine serum and 100 µg gentamicin/mL, referred to as complete DMEM. Isolated tendons were cut into 5 mm lengths and each tendon explant received a surgical nick across half its diameter, before being placed on a Gelman Sciences

filter membrane (Pall Corp. Port Washington, NY). The membrane was placed upon a stainless steel mesh screen suspended over the central well of a 60 mm Falcon #3037 organ culture dish (BD Biosciences, Lincoln Park, NJ). The central well of the organ culture dish was filled with 1.0 mL of complete DMEM, and surrounding outer moat had 2 mL of sterile water to maintain a humid environment. The complete organ culture set-up is presented in Fig. 1. Surface tension kept the tendon explants covered with a thin film of culture medium that allowed free gas exchange between the ambient air and tendon explant. FTFS were introduced to wounded tendons by directly pipetting 1 μ L of a suspension of FTFS onto the surface of tendon explants. Tendons were maintained in an incubator set at 37°C with 5% CO₂ in a water saturated atmosphere. At 24 hours tendon explants were harvested, fixed in 4% paraformaldehyde for 5 min. Fixed tendon explants were transferred to glass slides, treated with a drop of GelMount (Biomedica, Foster City, CA) and a cover-slip placed on top. Specimens were viewed with a Zeiss Axiovert 135 Fluorescent microscope (Zeiss, Thornwood, NY) with appropriate fluorescent filters and images taken with a Cool Snap FX digital camera (Roper Scientific, Ottobrunn, Germany).

To follow the progress of FTFS localization, with organ cultured wounded tendon explants, a group of explants were harvested and processed for fluorescence microscopy at 4, 18, and 24 hours after introducing FTFS; in all remaining studies, explants were harvested at 24 hours. The effects of high concentrations of antibiotic on the localization of FTFS with nick wounded tendon explants was investigated with neomycin sulfate as well as gentamicin at 100 μ g/mL in culture media for 24 hours. Cytochalasin D at 10 μ g/mL has been shown effective in preventing the polymerization of globular-actin into filamentous-actin, adverting actin-myosin generation of contractile forces.⁸ Colchicine at 10 μ g/mL inhibits the polymerization of tubulin monomers into microtubules, which are involved in aligning collagen fibers in tendon development.⁹ Cyclohexamide at 10 μ M, shown as an effective inhibitor of protein synthesis,¹⁰ was evaluated in altering the pattern and or accumulation of FTFS at wound edges of nick wounded tendon explants. All agents were purchased from (Sigma-Aldrich Co., St Louis, MO).

RESULTS

An aliquot of supernatant from the 90 day antibiotic extraction procedure was subjected to TEM examination and presented in Fig. 2. The negatively stained TEM micrograph revealed a FS with a collagen banding pattern, which was consistent with the morphology of an isolated collagen FS with a tapered end and a blunted end.¹¹ Negatively stained debris was present in the figure, which is a component of the supernatant fraction of the FS extraction from embryonic tendon suspended in gentamicin.

Nick wounded 14-day-old chick embryo tendon explants viewed with a fluorescence microscope at 4 hours after introducing FTFS showed red fluorescence uniformly distributed along the length of the tendon explant as well as along the explant wound edges, see Fig. 3, *above left*. At 18 hours there was a shift in fluorescence intensity, where greater fluorescence was localized at the wound edges and less intensity along the length of the tendon explant, see Fig. 3, *above right*. At 24 hours (Fig. 3, *below left*) the intensity of fluorescence at the wound edge overshadowed the intensity of fluorescence along the tendon surface. The single introduction of FTFS to wounded tendon explants generated this sequence pattern of fluorescence intensity, which after 24 hours remained constant for the next 2 days (figure not shown). After FTFS had accumulated at wound edges, they lost their ability to abandon wound edges and return to the tendon surface. In 24 hours an irreversible translocation of added FTFS had occurred, where FTFS on the surface of an explant had accumulated at wound edges. There was a rapid and dynamic change in the localization of

added FTFS from a uniform distribution along the length of the tendon explant to the limited aggregation at wound edges.

The isolation of FS from intact embryonic isolated tendons involved a long incubation time with a high concentration of gentamicin antibiotic. What changes with the pattern of fluorescence localization in the tendon explant model would occur by introducing antibiotics such as neomycin and gentamicin to tendon explants? The pattern of fluorescent FTFS translocation and accumulation in antibiotic treated wounded embryonic tendon explants was investigated with neomycin sulfate and gentamicin at 100 µg/ml. As shown in Fig. 4 *above* at 24 hours, the untreated tendon explants had a major portion of added FTFS accumulated at wound edges. With neomycin at 100 µg/mL, the intensity of fluorescence at wound edges was less than the fluorescence intensity along the length of the tendon explant, see Fig. 4, *below*. Substituting gentamicin at 100 µg/mL for neomycin, produced the same result as shown in Fig. 4, *below* (figure not shown).

The accumulation of FTFS at wound edges required their translocation along the tendon explant surface, followed by their entrapment at wound edges. This model suggested that tendon fibroblast locomotion forces were responsible for the transporting FTFS along the surface of the tendon explants. The cytoskeleton components of microfilaments and microtubules have been documented in generating cell locomotion forces. If cell locomotion forces play a role in the translocation of FTFS, then disrupting either microfilaments or microtubules would be expected to retard or eliminate the accumulation of FTFS at wound edges and no change in fluorescence intensity along the surface of the tendon explant. Cytochalasin D at 10 µg/mL, which disrupts microfilaments, prevented fluorescent FTFS accumulation at wound edges, see Fig. 5, *above*. The inclusion of colchicine at 10 µg/mL, which disrupts microtubules, prevented the accumulation of FTFS at wound edges see Fig. 5, *center*. Disruption of microtubules blocked FTFS accumulation at the wound edges.

Is new protein synthesis required for the translocation of FTFS in tendon explants and the accumulation of FTFS at wound edges? Treating nick wounded tendon explants for 24 hours with 10 µg/mL cyclohexamide, a protein synthesis inhibitor, did not alter the accumulation of FTFS at wound edges, see Fig. 5, *below*. The cyclohexamide treated tendon explants produced the same fluorescence pattern as untreated, control tendon explants. New protein synthesis was not necessary in tendon explants for the accumulation of FTFS at nick wound edges.

DISCUSSION

Here the definition of “translocation” is the actual transfer of the collagen FS from the surface of the tendon explant to the wound edges, whereas, “accumulation” is defined as the fixation of FS at wound edges. At 24 hours the introduction of fluorescent tagged preformed collagen, FTFS, to nick wounded tendon explants in organ culture produces a greater, localized, intense fluorescence at wound edges. The accumulation of FTFS at wound edges results from their passage along the surface of the tendon explant and entrapment within wound edges. The assumption is intrinsic tendon repair involves the initial release of FS from the damaged collagen fibers at the injury site, later followed by their reincorporation along with newly synthesized FS into healing wound edges.⁷ The organ culture tendon explant scheme utilized in this study facilitates the investigation of the reincorporation of FTFS into wounded tendons.

The regeneration of the repaired severed tendon by intrinsic tendon repair is dependent upon the availability of free FS in the area adjacent to the injury site. It is proposed that initially *in vivo* FS are released from the frayed ends of damaged tendon and their loss from the wound

site is responsible for the early translucent appearance at the gap edges early in the healing of a severed tendon.¹² There is evidence to support the notion that intact functional tendon fibroblast cytoskeletal components of microfilaments and microtubules are integral for intrinsic tendon repair. The cyclohexamide treated injured tendon explants; demonstrate that the early in tendon repair new protein synthesis is not essential for either FTFS translocation or accumulation at wound edges.

It is established in embryonic fibrillogenesis that collagen FS are generated within the extracellular compartments, crypts, on the surface of synthetic tendon fibroblasts.¹ It is proposed that FS are initially released from the frayed ends of damaged tendons¹² and later in the intrinsic tendon repair process are reattached to the frayed ends of healing tendon fibers.⁷ FS are 10.5 μm in length,¹¹ which makes it reasonable to assume that FS are transported along the surface of fibroblasts adjacent to the injury site, rather than FS ingested by fibroblasts that migrate to the wound edge and release them there.

The addition of antibiotics to the injured tendon explants shows the inhibition of FTFS accumulation at wound edges. It is not clear whether the inhibition of FTFS accumulation at the wound edges is a disruption of their translocation; inhibition of trapping at the wound edges site. The speculation is the disruption of FS bonding to a growing collagen fiber is involved. Since the incubation of native embryonic tendon in high concentrations of antibiotic was used to free FS from intact tendon explants, it suggests the inhibition of accumulation as the likely mechanism for failure to trap FTFS at frayed ends of nick wounded tendon explants. When giving antibiotics to patients undergoing tendon repair, should there be a concern that local concentrations of antibiotic at the wound site may be detrimental to intrinsic tendon repair. Extrinsic tendon repair involves increased synthetic activity with an increased vascular blood supply. On the other hand an increase in local vascularization is not a component of intrinsic tendon repair. The nonappearance of the classic wound healing process is a hallmark of intrinsic tendon repair. Hence, the absence of increased vascularization at the intrinsic tendon repair site diminishes the possibility of stockpiling antibiotic at that repair site.

FTFS translocation is their actual movement by the cells populating the surface of the tendon explant. With the nick wounded tendon explant repair model, the majority of FTFS translocation and accumulation at the wound edges occurs within 24 hours. The translocation component of intrinsic tendon repair can be accomplished through the random movement of FTFS. Their capture at wound edges has the potential to direct the movement of FTFS. The capture of FTFS at wound edges reduces their density in the immediate area adjacent to the wound edge. That local change in FTFS density creates a new equilibrium from the random movement of FTFS over the tendon surface. The continuing siphoning off of FTFS at wound edges directs the recruitment of distant FS to the wound edges. Here experimental evidence shows a role for microtubules and microfilaments in moving FTFS along the explant surface to the wound edges. Microtubules participate in the intracellular processing of procollagen to collagen. In these experiments it is demonstrated that they play a role in the processing of FS into tendons undergoing intrinsic repair. In the compaction of collagen, demonstrated in the fibroblast populated collagen lattice contraction model, the functional activities of both microfilaments and microtubules are required.¹³ Here experiments suggest that fibroblast tractional forces and not protein synthesis are important for the initial steps in intrinsic tendon repair. An argument can be made that the reutilization of released FS is advantageous for tendon repair.

The fetal tendon organ culture explant model demonstrates the possible involvement of FS in intrinsic tendon repair, utilizing simple components and conditions. A clearer understanding of the mechanisms of translocation and accumulation of FS in intrinsic

tendon repair is the focus of future studies. A better comprehension of mechanisms involved with intrinsic tendon repair may identify possible new approaches to promote a regenerative intrinsic repair in tissues other than tendon.

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Fig. 1.

The organ culture tendon explant setup is presented. A cut section of 14-day-old chicken embryo tendon is located on the surface of Gelman Sciences filter membrane that is placed on a stainless steel mesh screen suspended over the central well of a 60 mm Falcon #3037 organ culture dish. The dish's central well is filled with 1.0 mL of complete DMEM, and the surrounding well or outer moat has 2 mL of sterile water. Magnification 12×

Fig. 2. A TEM of negatively stained collagen fibril segment. A typical fibril segment is shown from the supernatant of a 10-day-old chicken embryo tendon subjected to 1 mg/mL of gentamicin antibiotic for 90 days at 4°C. Please note a collagen banding pattern within the central region of the FS as well as one end is tapered and the other blunted. Also included in the figure is negatively stained debris. Magnification 62,000×.

Fig. 3.

Changes in the fluorescence pattern of nick wounded tendon explants over time. FTFS were incubated with nick wounded tendon explants. (*Above left*) at 4 hours fluorescence intensity was uniform along the length of the explant with minimal increased intensity at wound edges. (*Above right*) at 18 hours there was a continued increase in intensity at wound edges with a loss of intensity along the length of the tendon explant. (*Below left*) at 24 hours fluorescence intensity along the length of the explant was minimal, while fluorescence intensity was mostly restricted to wound edges. The 24 hour fluorescence pattern (*Below left*) represents the standard fluorescence pattern for nick wounded tendon explants accumulation of FTFS at wound edges. (*Below right*) was the typical fluorescence pattern that appeared at the ends of untreated tendon explants at 24 hours, which showed the accumulation of FTFS was at all wound edges regardless of their location at the nick wound or the end of the tendon explant. Magnification 10 $\times$.

Fig. 4. High doses of antibiotic effect upon fluorescence with nick wounded tendon explants. (*Above*) an untreated, control nick wounded explant shows fluorescence intensity restricted to wound edges. (*Below*) compared to control tendon explants, tendon nick wounded explants that received 100 µg/mL of neomycin, failed to accumulate FTFS at wound edges. Magnification 10×.

Fig. 5. Pattern of fluorescence in nick wounded tendon explants treated with an inhibitor of microtubules, microfilaments or protein synthesis. (*Above*) at 24 hrs a typical explant treated with the microfilament actin polymerization inhibitor cytochalasin D, failed to accumulate fluorescence at wound edges. (*Center*) a typical explant treated with the microtubule inhibitor colchicine, failed to accumulate fluorescence at wound edges. (*Below*) a typical explant treated with the protein synthesis inhibitor cyclohexamide showed a fluorescence pattern similar to controls with accumulation of fluorescence exclusively at wound edges. Magnification 10 $\times$.