

Study on Epitenon Fibroblast Migration after Incising and Suturing the Tendon with Modified Kessler in New Zealand Rabbits

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Abstract

Introduction: In our study, we present a direct staining method of epitenon fibroblasts with the dye Dil (1,1'-dioctadecyl -3,3,3',3'-tetramethylindocarbocyanine percholate) using an animal model, and furthermore we observe the migration of these cells. Our methodology tries to imitate what actually happens in clinical practice, as far as possible.

Material and methods: In 36 New Zealand rabbits, the flexor digitorum profundus tendon of right anterior leg was pulled out of its sheath, incised and sutured with modified Kessler. The tendon was stained with the dye Dil and placed back to its sheath, where it was fixed distally. Tendon specimens were taken from 6 rabbits and examined under fluorescence microscopy at 1, 3, 5, 7, 14 and 28 days postoperatively. In order to evaluate our results, the fibroblasts' migration was divided into 4 phases: first phase, all the stained cells are on the tendon surface; second phase, there is only sporadic migration of fibroblasts under the tendon surface; third phase, there is massive migration of cells below the tendon surface; fourth phase, the cells are deeper into the tendon substance.

Results: The majority of epitenon's fibroblasts in the first 24 hours were found to be in the first phase (all stained cells were at the tendon's surface and only some cell clusters were found to be in the second phase). On the 3rd day, the majority was found to be in the third phase (massive migration under tendon's surface), and on the 5th day at the fourth phase (deeper in endotenon). No significant progress in cell migration was noticed the following days.

Discussion: After tendon trauma, the epitenon fibroblasts' migration starts early in the first day and peaks under the tendon's surface on the third day. By the 28th day, nearly all fibroblasts have moved in tendon's core substance.

Keywords: Tendon; Healing; Epitenon; Fibroblast; Migration; Dil; Kessler

Introduction

The issue of tendon healing concerns the scientific community for more than 50 years. Initially, Skoog and Peterson [1] at 1954 proposed that tendons did not have the ability for intrinsic healing and that cell migration from the surrounding tissue is necessary. Ten years later, Eiken et al. [2] suggested that intrinsic healing was possible, and that was confirmed by Lundborg and Rank [3]. In 1998, Chang et al. [4] proved with immunohistochemical methods that there is a combination of intrinsic and extrinsic mechanisms in tendon healing. It is still not clear which mechanism dominates, but nowadays, the existence of a combination of intrinsic and extrinsic healing that depends on several factors like wound type, tendon location, blood supply and biomechanics [5] is generally accepted.

The tendon is a connective tissue and consists of multiple parallel fibers of collagen in the extracellular matrix. It is composed of 86% collagen, 1-5% proteoglycans, 2% elastin and few other cells most of them fibroblasts. Tendon structure allows transfer of great load between muscle and bone. Furthermore, tendon oxygen consumption is 7.5 times less than skeletal muscle. Due to this low metabolic rate and the capacity to produce energy with anaerobic methods, tendons are able to transfer load and keep tension for long periods avoiding ischemia and necrosis. Because of this low metabolic rate, the healing process is very slow after trauma [6].

The tendon healing process starts immediately after trauma. This process is divided into three overlapping phases. The first phase (inflammatory or hemostatic) starts immediately after trauma and lasts up to 14 days. There is hematoma formation, due to vessel disruption, that organizes into a fibrous clot. Leukocytes and macrophages attracted from factors secreted from the histiocytes, migrate to the lesion area. The macrophages secrete factors that stimulate fibroblasts and promote microangiogenesis. Epitenon cells proliferate and migrate to the inflammatory area. A fibrin network is created. At this phase, the strength of tendon reconstruction is fully dependent on suture quality and the suturing method. The second phase (proliferative or fibroblastic), lasts 2-6 weeks, and there is great concentration of collagen type I produced mostly from collagen type III conversion. The fibrin network expands, and the randomly located collagen fibers are

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organized gradually along the tendon axis, bridging the tendon gap. Clinically, reconstruction strength depends mainly on the type and quality of the sutures. The third phase, (reconstructive-maturing), lasts several months. The collagen fibers are properly oriented bridging the tendon gap, and the collagen continues to convert from type III to type I, but fibroblast and collagen production is diminished. Studies from Gomez [7,8], Buckwalter and Hunziker [9], show that nearly one year is needed to restore the function and strength of the tendon back to normal.

Gelberman et al. [10] has shown that immediately after trauma the epitenon cells show greater activity and chemical production than endotenon cells and inversely, an increased apoptosis of the endotenon cells is observed. Later, during the next weeks, this ratio is reversed. McGrouther et al. [11] and other investigators reached to similar results by using mainly immunohistochemical methods and lately using the Dil dye, which stains the fibroblasts' DNA. Healing depends on the type and origin of cells, cell activation and proliferation, and the time of moving into the trauma area. Only few studies describe the cell response during the healing process *in vivo*.

Purpose

It is generally accepted that zone II tendon reconstruction is a challenge for the surgeon. The surgical outcome can be compromised because of the formation of adhesions or because of re-rupture at the reconstruction site. The benefit would be major if we could promote healing and decrease adhesions by decoding the mechanisms of tendon healing. In our study, we evaluate the cell response after tendon trauma by direct observation of the epitenon fibroblast migration using the Dil dye (1,1'-dioctadecyl -3,3,3',3'-tetramethylindocarbocyanine percholate) on an animal model. Our methodology resembles the clinical practice. We study what actually happens at the site of tendon healing after complete laceration and suturing with modified Kessler's technique. This study is a continuation to a previous study evaluating the response of the tendon sheath cells after injury by staining with Dil [12].

Material and Methods

In order to study the activity and the migration of epitenon fibroblasts, we used the non-toxic, fluorescent, lipophilic dye 1,1'-dioctadecyl -3,3,3',3'-tetramethylindocarbocyanine percholate (Dil) (Molecular Probes Inc., Eugene, OR). This dye is widely used for tracking cells and tissues in laboratory specimens up to 4 weeks and *in vivo* up to one year. It infiltrates the cell via lateral diffusion in the plasma membrane, where it binds to DNA, at a rate of 0.2-0.6 mm per day in fixed specimens, and up to 6mm per day in living tissue, due to active dye transport. In general, the dye does not transfer from cell to cell, although some transfer may take place when the cell membrane is disrupted, as occurs after sectioning.

The dye was received in the form of microcrystals; therefore, a sterile phosphate buffer solution (500 ml 136 g/l KH_2PO_4 + 29,5 ml 1 M NaOH diluted in 1000 ml H_2O) was created with pH 7.0 at an environmental temperature. Previous studies [12-14] propose the suitable concentration of the Dil solution and proper application time on the epitenon.

General anesthesia was performed on 36 New Zealand rabbits aging 12-18 months by intramuscular administration of ketamine (Narketan 10, Vetoquinol) and pentobarbital (Dolethal, Vetoquinol). In supine position, after shaving the front limbs and disinfecting the

skin with povidone iodine 10% solution (Betadine, Lavipharm), sterile dressings were placed and local anesthetic xylazine hydrochloride 2% (Xylaject 2%, Dopharma) was injected into the skin incision areas. A transverse plantar incision of 1-1.5 cm was performed on right front leg at the level of the ankle. Under the flexor digitorum superficialis (FDS) tendon, that was moved aside, the flexor digitorum profundus (FDP) was identified and exposed with hemostatic forceps. Its size, on that level, is similar to many human flexors, although it has higher consistency. FDP was sectioned and loosened sutures were placed at both sides (fixing Kessler). After that, a new skin incision was performed 2-3 cm proximally and slightly medially to the first incision. The muscle belly of FDP was identified and separated from the surrounding tissues. By gentle traction, the tendon was pulled out of its sheath while the sutures were left in place for later easy repositioning of the tendon. A complete transverse section of the exposed tendon was performed, and the two parts were sutured by using (modified Kessler) prolene 4/0 for the core and prolene 6/0 for the running suture of the epitenon with the aid of surgical loupes (Figure 1). Subsequently, the tendon was carefully wrapped in a gauze impregnated with Dil solution, so as to avoid leakage in the wound and the entire portion of the tendon surface was in contact with the gauze for 5 minutes. The area was covered to prevent drying of tissues. The gauze was removed, and the tendon was washed thoroughly with normal saline, dragged back in the sheath and fixed distally with a Kessler type suture. For closure of the surgical wounds nylon 3/0 was used and iodine antiseptic solution was applied on the skin. Intraoperative injection of cefuroxime was administered intramuscularly, and the rabbits were left in cages without any leg immobilization or any wound dressing. The same procedure was applied for the left front limb that was used as control, but without transecting the intrasynovial part of the tendon.

Specimens of the flexor tendons 1-1, 5 cm long were taken from 6 animals at days 1, 3, 5, 7, 14 and 28. The samples were stored in liquid nitrogen, transferred to histopathology laboratory and 8 μm sections, 4 from each specimen, were taken with a cryostat tungsten blade. Totally, 144 sections were studied under ultraviolet microscopy using a special optical filter with spectrum Ex 546 nm and Em 573 nm to visualize Dil. Images were taken at magnification x100, x200, x400 by a camera connected to the microscope and stored in a computer. To evaluate the outcomes, the fibroblast migration was divided into four phases. In the first phase, all the stained cells are on the tendon surface. In the second phase, there is only sporadic migration of fibroblasts under the tendon surface. In the third phase, there is massive migration of cells below the tendon surface. Finally, in the fourth phase, the cells are, deeper, in tendon substance.

Results

Two rabbits were excluded, one due to inability to take good quality



Figure 1: Shows the flexor tendon reconstruction with modified Kessler technique on rabbit's front leg and the sutures for fixing the tendon distally.

cryosections and the other because of a suturing failure, few days after the surgery.

On the 1st day after surgery 90% of samples were categorized at 1st phase of cell migration since nearly all stained cells were observed on tendon surface and only in 10% of the specimens, the fibroblasts were found under the tendon surface (2nd phase). On the 3rd day 75% of samples were found at 3rd phase and 25% at 2nd phase. On the 5th day 12.5% of samples were in 1st phase, 25% in 2nd phase and the majority had migrated massively below the tendon surface (3rd phase), (Figure 2). On the 7th day 10% of specimens were categorized in 2nd phase, 20% in 3rd phase and in most of them (70%) stained cells were found deeper in the endotenon (4th phase). On the 14th day, more fibroblasts were gathered in the endotenon and 80% of specimens were classified in the 4th phase, and only a few in the 2nd and in the 3rd phase. On the 28th day, all specimens were found with scattered cells uniformly in the core substance of the tendon (4th phase), (Figure 3).

The contralateral limbs were used as controls. The results for the first day were similar, in particular, 90% of the specimens were in the 1st phase, and 10% were in the 2nd phase of cell migration. On the 3rd day 87.5% of specimens were in the 1st phase and only 12.5% in the 2nd phase. On the 5th day 50% were in the 1st phase and 50% in the 2nd phase. These numbers remained the same for the next period, and the 3rd and 4th phases were not observed at all (Table 1 and Chart 1).

Statistical Analysis

Simple linear regression analysis was used to check the correlation between the number of days after the tendon injury and the phase of the cells migration using the software of SPSS 16.0. Based on that analysis we estimated that the r square equaled 0.562 and the b1 equaled 5.745 for p=0.00. That means there is a weak but statistically significant correlation between the number of days after surgery and the phase of cell migration.

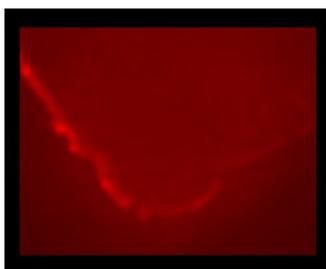


Figure 2: Ultraviolet photomicrograph (x100) at 1st day showing cell clusters stained with Dil on the tendon's surface (left hand side) and the beginning of cell invasion in the area of the incision.

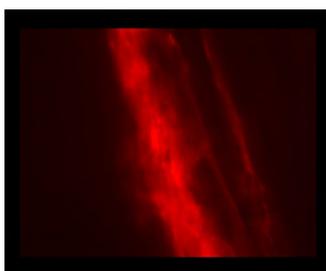


Figure 3: Ultraviolet photomicrograph (x200) showing the massive migration of Dil stained cells under tendon's surface on the 5th day.

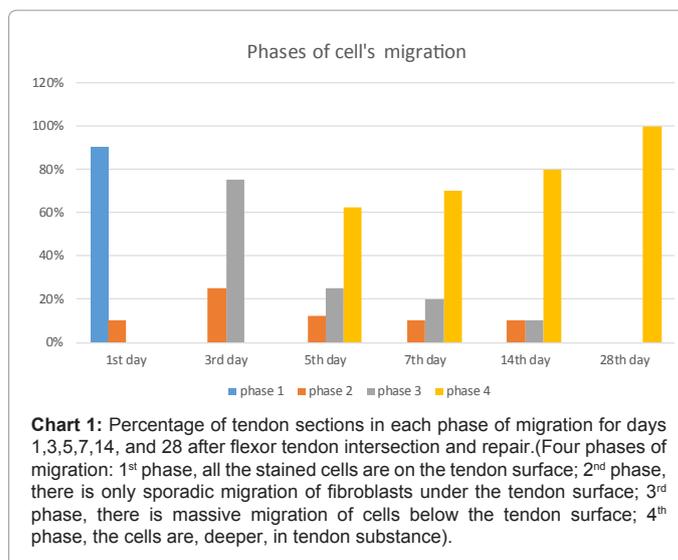


Chart 1: Percentage of tendon sections in each phase of migration for days 1,3,5,7,14, and 28 after flexor tendon intersection and repair.(Four phases of migration: 1st phase, all the stained cells are on the tendon surface; 2nd phase, there is only sporadic migration of fibroblasts under the tendon surface; 3rd phase, there is massive migration of cells below the tendon surface; 4th phase, the cells are, deeper, in tendon substance).

Tendons sectioned and sutured with modified Kessler				
	1 st phase	2 nd phase	3 rd phase	4 th phase
1 st day	90%	10%		
3 rd day		25%	75%	
5 th day		12.5 %	25%	62.5%
7 th day		10%	20%	70%
14 th day		10%	10%	80%
28 th day				100%
Control group				
1 st day	90%	10%		
3 rd day	87.5%	12.5%		
5 th day	50%	50%		
7 th day	50%	50%		
14 th day	50%	50%		
28 th day	50%	50%		

Table 1: Tendon specimen percentage for each time period and migration phase of fibroblasts.

Discussion

Most recent studies, dealing with tendon healing, support the existence of a complicated repair mechanism during which epitenon and sheath fibroblasts are more active than endotenon fibroblasts [12]. Furthermore, at first week after injury endotenon fibroblasts seems to undergo apoptosis [9-11]. Fibroblast migration has been studied *in vitro* [15-18] and *in vivo* [19-21] and it was found that epitenon fibroblasts are acting early at the initial stage of healing, and their migration on living animal models starts on the 3rd day (almost a week earlier than in the *in vitro* model). This delayed healing is partially attributed to lack of motion. In our animal model, migration of fibroblasts started earlier peaking on the 3rd day, while on the 5th day the stained cells were found in the core of the tendon. On the 28th day, nearly all cells had intruded the substance of the tendon giving a uniform image of stained cells in the core, but almost none was observed on the surface of the tendon (Figure 4).

The Dil dye has been successfully used at the past for tracking cells on different animal models and although in some studies [13] it had short lasting action, in our model, it stained the cells for longer time intervals. A pilot study [12] that we performed in order to determine

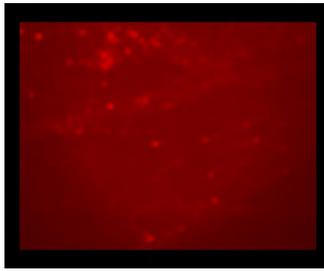


Figure 4: Ultraviolet photomicrograph (x400) showing scattered migrating fibroblasts in the tendon's core substance on the 28th day.

the optimal dilution for Dil showed that it was possible to detect fibroblasts on a living rabbit up to 84 days later. We believe that further studies could be done using this useful dye.

In the control specimens (the tendons of the left front leg without intrasynovial tendon injury) we observed a slight increase of the migrating epitenon fibroblasts. This increase was attributed to the inflammatory stimulation, due to the nearby extrasynovial tendon wound done to pull-out the flexor and to fix it later with type Kessler suturing (see Material and Method). The presence of some stained cells just under the surface of the tendon could be explained by dye diffusion, or we could presume the existence of a constant membrane diffusion mechanism of some fibroblasts from the epitenon towards below its surface and vice versa.

Tendon healing, especially in the hand, is a slow repair process, and that results in inferior scar tissue formation and many times inability of full range of motion. Despite the progress of the suturing material quality, the different suture techniques and the rehabilitative protocols followed by hand therapists, a significant number of patients is suffering from complications such as re-rupture and adhesion's formation that limit the hand function [22-25]. Good understanding of the healing tendon mechanism is crucial to develop new strategies that will increase strength of the repair tissue and finally will result in a better clinical outcome. Fibroblasts seem to play a critical role on that. It is hypothesized nowadays that if they could be stimulated by growth factors such as basic fibroblast growth factor (bFGF) [26] the healing process would possibly be faster and stronger, with fewer adhesions at the reconstruction site due to early mobilization, and smaller rupture risk. Only a few studies deal with the specific role of fibroblasts on tendon healing [13,14] and just lately researchers showed the morphological and biomechanical parameters of healing on tendons reconstructed with modified Kessler's suture. They concluded that further research on biology and dynamics of tendon healing is necessary in order to develop new strategies on tendon reconstruction [27-29].

In our study, we have chosen modified Kessler's technique because it is reproducible, relatively easy, with good strength and providing smooth gliding surface [30,31] without disturbing the blood supply of the tendon [7]. We suggest a methodology and an animal model that resembles what actually happens in clinical reality, since tendon healing and fibroblast migration is studied after reconstructing the injured tendon with a widely used suturing method. This realistic method could be used by other investigators to study factors and biomechanical methods for better healing and fewer adhesions. We propose this model for further research due to the proved importance and early action of endotenon fibroblasts on tendon healing, and due to the limited problems that we faced in terms of infection, size, and biomechanical issues.

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