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Review

Biology and physiology of tendon healing

Christelle Darrieutort-Laffite^{a,b,*}, Frédéric Blanchard^b, Louis J. Soslowsky^c,
Benoit Le Goff^{a,b}

^a Service de rhumatologie, CHU de Nantes, Nantes, France

^b Oniris, Regenerative Medicine and Skeleton, RMeS, UMR 1229, Inserm, CHU de Nantes, Nantes université, 44000 Nantes, France

^c Department of Orthopaedic Surgery, McKay Orthopaedic Research Laboratory, University of Pennsylvania, Philadelphia, PA, USA



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ABSTRACT

Tendon disorders affect people of all ages, from elite and recreational athletes and workers to elderly patients. After an acute injury, 3 successive phases are described to achieve healing: an inflammatory phase followed by a proliferative phase, and finally by a remodeling phase. Despite this process, healed tendon fails to recover its original mechanical properties. In this review, we proposed to describe the key factors involved in the process such as cells, transcription factors, extracellular matrix components, cytokines and growth factors and vascularization among others. A better understanding of this healing process could help provide new therapeutic approaches to improve patients' recovery while tendon disorders management remains a medical challenge.

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1. Introduction

Tendon disorders affect people of all ages, from elite and recreational athletes and workers to elderly patients. Tendon injuries vary from acute traumatic ruptures to chronic overuse and degenerative tendinopathy. The mechanism of injury for a given tendinopathy can vary from region to region, and from patient to patient. The dominant mechanisms for many tendinopathies are tensile overload and age related degeneration [1].

On the basis of animal studies, it has been shown that three distinct phases regulate the physiology of tendon healing after an acute injury: an inflammatory phase, a proliferative phase, and a remodeling phase [2,3]. The inflammatory stage initiates the response to injury throughout the first week. The process is characterized by the development of a fibrin clot to stabilize the site, and migration of neutrophils and macrophages. Then, matrix-producing tenocytes localized to the injury site start synthesizing collagen and other extracellular matrix (ECM) components throughout one to four weeks following injury. Cellular proliferation and matrix production occur during this stage but the collagen produced is not well-organized. The final stage of remodeling and maturation begins around four weeks after the injury and continues

until the tissue is repaired. During this phase, the ECM is remodeled to create a more organized structure through collagen turnover, realignment, and formation of collagen cross-links. Cell density and vascularity decrease as the tissue repairs. However, animal studies have shown that natural healing leads to tendon biomechanical properties that fail to match normal levels at up to twelve months after injury, depending on the model [4,5].

The objective of the review was to summarize available data on tendon healing process and its key regulators such as tendon cells, transcription factors, ECM components or cytokines and growth factors and to discuss factors involved in altered wound healing contributing to tissue degeneration. Better understanding of tendon physiology, degeneration and healing processes will help to develop new treatments for tendon regeneration.

2. Tendon cells

The main population of cells in tendons are tenocytes. Tenocytes secrete the ECM and growth factors to maintain tendon homeostasis. Cells distributed in the interfascicular matrix area are termed interfascicular tenocytes, while the cells distributed in the fascicle area are termed intrafascicular tenocytes. As with many other tissues, tendons also contain a population of stem/progenitor cells called tendon stem/progenitor cells (TSPCs). These cells display mesenchymal stem-cell (MSC) – like properties and showed a differentiation potential toward the osteoblast, adipocyte, tenocyte and chondrocyte lineages. They express classical MSC – associated sur-

* Corresponding author. Department of Rheumatology, CHU of Nantes, 1, place Alexis-Ricordeau, 44000 Nantes, France.

Adresse e-mail : christelle.darrieutort@univ-nantes.fr (C. Darrieutort-Laffite).

face markers such as Stro-1, CD44, CD73, CD90, CD105 and CD146 [6–9] and are located within a fibromodulin- and biglycan-rich niche [8]. Cells expressing both tendon- and stem-cell associated markers have also been identified in the perivascular niche of supraspinatus tendons [10]. More recently, single-cell surface proteomics confirmed the presence of this perivascular niche with a tendon cell cluster expressing high levels of CD90 and CD146 [11]. In Achilles tendon, Mienaltowski et al. described two distinct stem/progenitor cell populations, either located in the paratenon or the tendon proper. Those cells were negative for the perivascular surface marker CD133 and display a differential expression pattern of tenomodulin and scleraxis [12]. They also displayed different potential for forming functional tendon-like tissue [13]. Taken together, these data suggest that TSPCs constitute heterogeneous groups of cells with distinct characteristics [14].

After tendon injury, cells from the surrounding peritenon proliferate, migrate and contribute to tenogenesis. Following patellar tendon injury, α SMA+ paratenon cells were the main contributors to the healing response. They extended over the defect space at 1 week and differentiated into Scx+ cells at 2 weeks, with concomitant increased collagen signal in the paratenon bridge [15]. Another study showed that a stem-cell Sca-1-positive and Scx-negative progenitor population display Scx induction, migrate to the wound site, and produce ECM to bridge the defect [16]. In Scx-deficient mice, the Sca-1-positive progenitor cells were able to migrate to the lesion but were unable to assemble the ECM to bridge the defect, thus highlighting the critical role of Scx in a progenitor cell lineage in wound healing of adult tendons. Recent data from combined genetic lineage tracing and single cell RNA-Sequencing (scrRNA-seq) confirmed that cells driving healing originate from the epitenon [17]. Among TSPCs, nestin+ TSPCs exhibited superior tenogenic capacity compared to nestin- TSPCs, and inactivation of nestin expression in TSPCs suppressed their clonogenic capacity and reduced their tenogenic potential, both in vitro and in vivo. So nestin seems to have a crucial role in TSPC fate and phenotype maintenance [18].

Finally, healing can be supported by cells coming from adjacent tissues such as muscles or bone. In adult skeletal muscles, a Scx+ cell subpopulations displaying an elongated morphology in vitro and expressing tendon markers (TNMD, THBS4, EGR1, and COLA1) but not the muscle-specific transcription factor MYOD1 has been identified. This suggests that adult muscles also contains a potential reservoir for tendon regeneration [19]. Otherwise, single-cell RNA-sequencing defined six resident cell types in the enthesis region, including a unique enthesis Gli1+ progenitor population able to enhance enthesis healing [20].

However, in order to fully assess the regenerative capacity of tendon stem-cells, we need to gain further insights into the *in vivo* identification of these tendon cells and how they are modulated by the local niche.

3. Transcription factors

After tendon injury, expression of genes encoding tendon-associated transcription factors, Egr1, scleraxis (Scx), Mohawk (Mkx), is upregulated.

3.1. EGR1 (early growth response 1)

Egr1 is upregulated during tendon development and seems to be important for tendon cell differentiation, collagen type I production, and can induce expression of scleraxis [21–23]. Egr1 is required for optimal wound healing and is also strongly connected to fibrosis in different tissues as an important mediator of transforming growth factor- β (TGF- β)-induced responses. However,

expression of Egr1 in healing tendons has not been associated with fibrosis or scar formation, but instead EGR1 has been shown to promote tendon repair [24]. Egr1 was required for the normal gene response following tendon injury in a mouse model of Achilles tendon healing. Forced Egr1 expression in MSCs promoted the formation of *in vitro*-engineered tendons [22]. It also promoted tendon repair in a rabbit model of rotator cuff injury through the BMP12/Smad1/5/8 signaling pathway. In Egr1^{-/-} Achilles tendon, the increase of Scx, Tnmd, Col1a1, Col1a2, Col5a1, Col6a1, Col14a1, Tenascin-C, and Decorin gene expression was drastically decreased showing that Egr1 is required for increased expression of tendon-related ECM genes and key tendon markers [25].

3.2. SCX

The transcription factor Scleraxis, is expressed throughout tendon development and plays essential roles in both embryonic tendon development and adult tendon healing. It is the most widely studied and the most well characterized factor utilized as a “tendon marker”. Although not exclusively expressed in tendons, Scx is a key regulator in tenocyte differentiation of embryonic stem-cells (ESCs) and mesenchymal stromal cells (MSCs). Mice lacking Scx exhibit a specific and virtually complete loss of tendons during development.

After injury, Scx expression has been shown to be significantly upregulated, playing a central role in tendon healing in adult animals [16,26]. Following injury, a paratenon cell population, Sca-1-positive and Scx-negative progenitor subpopulation, display Scx induction before migrating to the wound site, and produce extracellular matrix (ECM) to bridge the defect [16]. Scx induction in the progenitors is initiated by TGF- β signaling. In Scx-deficient mice, migration of Sca-1 positive progenitor cells to the injury site was still observed but ECM assembly to bridge the defect was impaired [16]. Mechanistically, Scx-null progenitors displayed higher chondrogenic potential and Scx-null wounds formed cartilage-like tissues that developed ectopic ossification. In addition, Scx expression is required for maintenance of myofibroblasts (α SMA+ cells) during tendon healing [27,28].

More recently, mouse models for Scx+ cell labelling helped us to better understand the specific role of Scx+ cells in tendon repair. In uninjured tendons, Scleraxis lineage (Scx^{Lin}) cells represented the majority of the total tendon cell population. Then, adult Scx^{Lin} cells localize within the scar tissue and organize into a highly aligned cellular bridge during tendon healing [29]. However, depletion of Scx^{Lin} cells prior to injury in the same model (transection and repair of the flexor digitorum longus tendon) did not impair healing. These data suggest that “new” Scx^{Lin} cells (e.g., those that express Scx in response to tendon injury, but that were not Scx^{Lin} at the time of depletion), may predominate during early healing [30].

3.3. Mohawk

Mkx is expressed in progenitor cells of muscle, cartilage, bone and tendon. It promotes tendon differentiation and prevents chondrogenic/osteogenic differentiation [31–33]. Mkx mutant mice showed severe tendon hypoplasia and Mkx-deficient tendons displayed reduced expression of tenomodulin (Tnmd) and ECM components decorin (Dcn) and fibromodulin (Fmod) [34,35]. Besides its role in tendon homeostasis, Mkx is involved in tendon healing. After a complete transverse section of mouse Achilles tendon, the implantation of Mkx-expressing-MSC sheets improved tendon repair [32].

Scx, Mkx and Egr1 are three transcription factors involved in tendon development, and they all have the ability to induce tenogenesis in stemcells. The implantation of stemcells producing any of these transcription factors improves tendon repair in animal

models for tendon injury [36]. However, the interactions between these transcription factors during tenogenesis remain not fully understood and could be different between *in vitro* and *in vivo* systems and between species. Other studies are still needed to elucidate this network fully.

4. ECM components

A tendon is a connective tissue structure mainly composed of collagen: 70 to 90% of tendon extracellular matrix (ECM) is composed of a hierarchical collagen structure. This high amount of collagen and its organized disposition with parallel fiber orientation provide resistance to deformation primarily in the fiber direction [37]. The subunits of collagen fibers are fibrils. Fibrils diameter can vary from a few nanometers to about 5000 nm. In adult tendons, the diameter of collagen fibrils exhibits a bimodal distribution. Specific inter-molecular cross-links bind adjacent microfibrils together, thereby keeping collagen fibrils stable. The remaining 10–30% of the ECM consists of a variety of non-collagenous proteins (elastin), proteoglycans (Dcn, biglycan [Bgn], lumican, Fmod, lubricin, aggrecan), and glycoproteins (Tnmd, Tenascin-C, Cartilage Oligomeric Matrix Protein [COMP], fibronectin). These non-collagenous ECM constituents have been suggested to contribute to tendon's characteristic quasi-static and viscoelastic mechanical behavior in tension, shear, and compression [37].

Tendon injury leads to a massive increase in the relative mRNA levels of genes encoding collagens (Col1a1, Col1a2, Col3a1, Col12a1, Col14a1) and tendon-associated molecules including Tnmd, tenascin (Tnc) and proteoglycans in animal models [38]. Among these ECM components, some are involved in regulating TSPCs functions. Bgn and Fmod, have been shown to be crucial in regulating the lineage fate of TSPCs, since their depletion in double knock-out animal led instead to bone-like tissues being formed [8]. Tnmd is essential for maintaining the self-renewal capacity and matrix remodeling capacity of TSPCs [14]. Overexpression of Tnmd in murine mesenchymal stem-cells (MSCs) inhibited their commitment towards the adipogenic, chondrogenic and osteogenic lineages, whilst promoting their tenogenic differentiation [39]. It is also required for prevention of adipocyte accumulation and fibrovascular scar formation during early tendon healing [40].

Biglycan and decorin are class I SLRPs. Decorin is the most abundant (about 80% of the total proteoglycan content). These proteoglycans are known to regulate collagen fibril assembly. The GAG side chain of two decorins respectively attached to two collagen fibrils can interact and form an interfibrillar bridge to connect the neighboring fibrils. Biglycan competes for the same binding site of collagen fibrils and is also involved in the maintenance of the fibril structure of collagen. After an injury, a transient increased expression of biglycan is observed while decorin expression is reduced. Mature (120 days old) *Bgn*^{-/-} mice and *Dcn*^{-/-} mice demonstrated attenuated early and late-stage healing, respectively [41]. Biglycan can also act as a signaling molecule that regulates inflammation [42]. Otherwise, healing tendons contain more elastin and are more compliant than intact tendons. The role of elastin in tendon healing and tissue compliance suggest a potential protective role of elastic fibers to prevent re-injuries during early tendon healing [43].

ECM remodeling is regulated by metalloproteinases (MMPs): MMP-1, -2, -3, -8, -9, -13, and -14 are involved in tendon damage [44]. They act through an imbalance with their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). MMPs, TIMPs, and ADAMTs (a disintegrin and metalloproteinase with thrombospondin motifs) are the main factors regulating the ECM network remodeling. MMP-9 and -13 mediate tissue degradation during the early phase of healing, whereas MMP-2, -3 and -14 mediate tissue degradation and later remodeling [45].

5. Cytokines and growth factors

A large panel of cytokines and growth factors are released by the injured tendons and adjacent tissues, including interleukins, tumor necrosis factor (TNF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), TGF- β , connective tissue growth factor (CTGF), epidermal growth factor (EGF), and insulin-like growth factor (IGF)-1 [38].

5.1. Cytokines

When an injury occurs, ECM degradation products formed from collagen, fibronectin, or biglycan were proven to be potential DAMPs that upregulate pro-inflammatory mediators like IL-1 β , TNF- α and IL-6 through NF- κ B activation. ECM-derived DAMPs have also been reported to be the ligands for pattern recognition sequences like toll-like receptors (TLRs). The inflammatory phase lasts three to seven days from the injury, and it is featured by a prevalence of inflammatory cells such as monocytes and macrophages [46]. In this phase, the inflammatory response to the injury elicits platelet activation and the formation of granulation tissue which allows cells to migrate from surrounding areas to the injured site. In animal models of tendon injury, gene and protein expression of IL-1 β was increased in the early stages of injury or healing for two weeks following the intervention. IL-6 influences immune function and is involved in tendon healing [47,48]. IL-6 is implicated in the early phase of tendon healing, promoting the increase of COL1A1 expression in tendons. In animal models of tendon injury, IL-6 gene and protein expression was increased from two hours to four weeks following the injury [45]. Other cytokines are involved in tendon healing: IL-4, IL-13, IL-8 or IL-21. These cytokines play a role in the early inflammatory phase of healing, regulating tenocyte proliferation, collagen synthesis and production of prostaglandin E2 (PGE2) [45]. In animal injury models, TNF α expression is also transiently increased from two hours to nine days following the intervention and decline two weeks later.

Inflammation could determine the fate of TSPCs as inflammatory signaling and mediators were shown to have effects on them. For example, PGE2 decreases the proliferation capacity of TSPCs, and induces their non-tenogenic differentiation [49]. IL-1 β could promote the motility of TSPCs and also cause phenotype loss of TSPCs, which is associated with altered expression of tendon-related genes [50]. Later, healing is characterized by a resolving inflammatory phase. At this stage, up-regulation of IL-6, IL-8 and IL-10 has been observed [50]. TSPCs play a regulatory role here by upregulating IL-10 expression through the JNK/STAT signaling pathway [51].

5.2. Growth factors

The main growth factors and their role in tendon healing are summarized in Table 1.

6. Immune cells

Although tendon was previously thought to have no immune cells, several studies now report the presence of both innate (macrophages) and adaptive (T cells) immune cell types within normal tendons [61]. The presence of immune cells in tendon suggests that these cells may function as first responders in the event of damage.

The early inflammatory phase (immediately after tendon injury) is associated with the release of interleukins and TNF α produced by pro-inflammatory M1 macrophages, whereas the secondary inflammatory response involves anti-inflammatory M2

Table 1
Main growth factors involved in tendon healing.

Growth factors	Effect on tendon healing	References
TGF β (transforming growth factor-beta)	·Involved in the initial inflammatory response, collagen synthesis, angiogenesis, and fibrosis/excessive scar formation TGF β 1: associated with the pathogenesis of excessive scar tissue formation. ·Disruption of TGF β 1 during healing reduced the extent of scarring, but this affected the mechanical strength of the tendon. Administration of TGF β 3 had beneficial effect on healing by accelerating the remodeling process	[52,53]
bFGF (basic fibroblast growth factor)	·Peaks in the early phase of healing Stimulation of the proliferation and differentiation of vascular endothelial cells During the proliferative phase, stimulation of fibroblasts to secrete type III collagen	[54]
PDGF (platelet-derived growth factor)	·Promotes tendon cell proliferation, collagen synthesis, and angiogenesis. ·Induction of tenogenic differentiation.	[55,56]
BMP (bone morphogenetic protein)	·BMP12: induced BM-MSC differentiation into tenocytes and improved molecular, organizational, and mechanical properties of healing tendon. BMP4: enhanced enthesis healing by promoting the regeneration of fibrocartilaginous enthesis and mineralization	[57–59]
VEGF (vascular endothelial growth factor)	·Promotes angiogenesis and activates the synthesis of other growth factors. Stimulates matrix metalloproteinases to degrade connective tissues to facilitate angiogenesis	[52]
IGF-1 (insulin-like growth factor)	·Supports cell proliferation, DNA and matrix synthesis, particularly collagen I	[60]

macrophages, which produce growth factors involved in neovascularization, such as VEGF, FGF and PDGF, and profibrotic factors, such as TGF- β and CTGF. After Achilles tear and repair, M1 macrophages accumulated in regions of newly formed tendon tissue 3 days following the injury and were still present 28 days. However, M2 macrophages slowly accumulated and became the predominant macrophage phenotype by 28 days [62]. This switch between M1 and M2 macrophages appears essential for adequate repair of tendon tissue. However, given the plasticity of these cells, it is possible that more diverse populations contribute to the healing process. The current development of transcriptomics will most likely help us to better define the macrophage populations involved in tendon healing.

7. Vascularization

Angiogenesis, the process of new blood vessel formation from existing blood vessels, is a key aspect of every repair process. Neoangiogenesis allows the supply of nutrients, oxygen, and healing factors as well as the clearance of cellular debris. Some studies indicated that uncontrolled vessel growth or impaired vessel regression could impair tissue healing, resulting in scarring and dysfunction. The number of supplying vascular branches significantly differs between various tendons. As tendons are moving tissues which are stretched by mechanical load, their vasculature must be compliant. Vessels form “curves” within their embedding endotenon tissue, a loose intra-tendinous tissue surrounding individual fascicles [63].

Neoangiogenesis in tendon healing requires the coordinated release of synergistic growth factors. VEGF expression has already been shown to be increased in injured Achilles tendons and its production after injury supports their role in tendon repair [64]. After patellar tendon injury, the number of vessels/mm³ was increased with a peak at day 3 then decreased at 2 weeks to remain unchanged until 20 weeks but still higher than that of the uninjured tendons [65]. After Achilles injury in aged rats, angiogenesis was reduced compared to adult animals and the delivery of VEGF caused an increase in vascular response to tendon injury and improved mechanical outcomes [66]. In addition, the delivery of anti-VEGF antibodies caused a temporary reduction in healing of rats Achilles

tendons [67]. These data suggest the crucial role of an increase in vascularization for proper healing.

8. Mechanical loading

Mechanical loading is essential for tendon development, homeostasis and repair. Physiological exercise increases collagen I turnover while overloading or underloading have negative effects on the tendon [68]. By stimulating tenocyte activity (cell proliferation and collagen synthesis/rearrangement), mechanical stimulation through controlled mobilization promotes tendon repair and remodeling, leading to increased tendon tensile strength and less adhesion than fixed healing tendon [69]. Contrary to what has been believed, mechanical loading has a stimulatory effect not only during the early remodeling phase but also when applied during the inflammatory phase [70]. Although beneficial in both phases, effects of loading are different when applied during the early or later phase of healing [24].

Two signaling pathways related to mechanical factors, TGF- β -SMad2/3 and FGF-ERK/MAPK, have been shown to play a role in tendon healing. Mechanical loading leads to an up-regulation of type III collagen mRNA expression and increased growth factors inducing cell proliferation and differentiation, and matrix production. Mechanical stimulation during tendon healing is also beneficial for the proper organization of collagen fibers [69].

The main steps of the healing process are summarized in Fig. 1.

9. Healing process and tendinopathy

Pathological features observed in tendinopathy consist in changes in the ECM characterized by a loss of collagen organization with deposition of additional proteins such as GAGs and type III collagen leading to inferior mechanical properties. In addition, tenocytes become rounder and more proliferative and increased neovascularization combined with neoinnervation are observed. The pathological process seems to often be initiated by repetitive tendon overload, leading to structural injury of the collagen fibrils. Under normal circumstances, early tendon matrix injury triggers an effective healing process; however, poor intrinsic healing ability of the tendon or lack of adequate recovery might lead

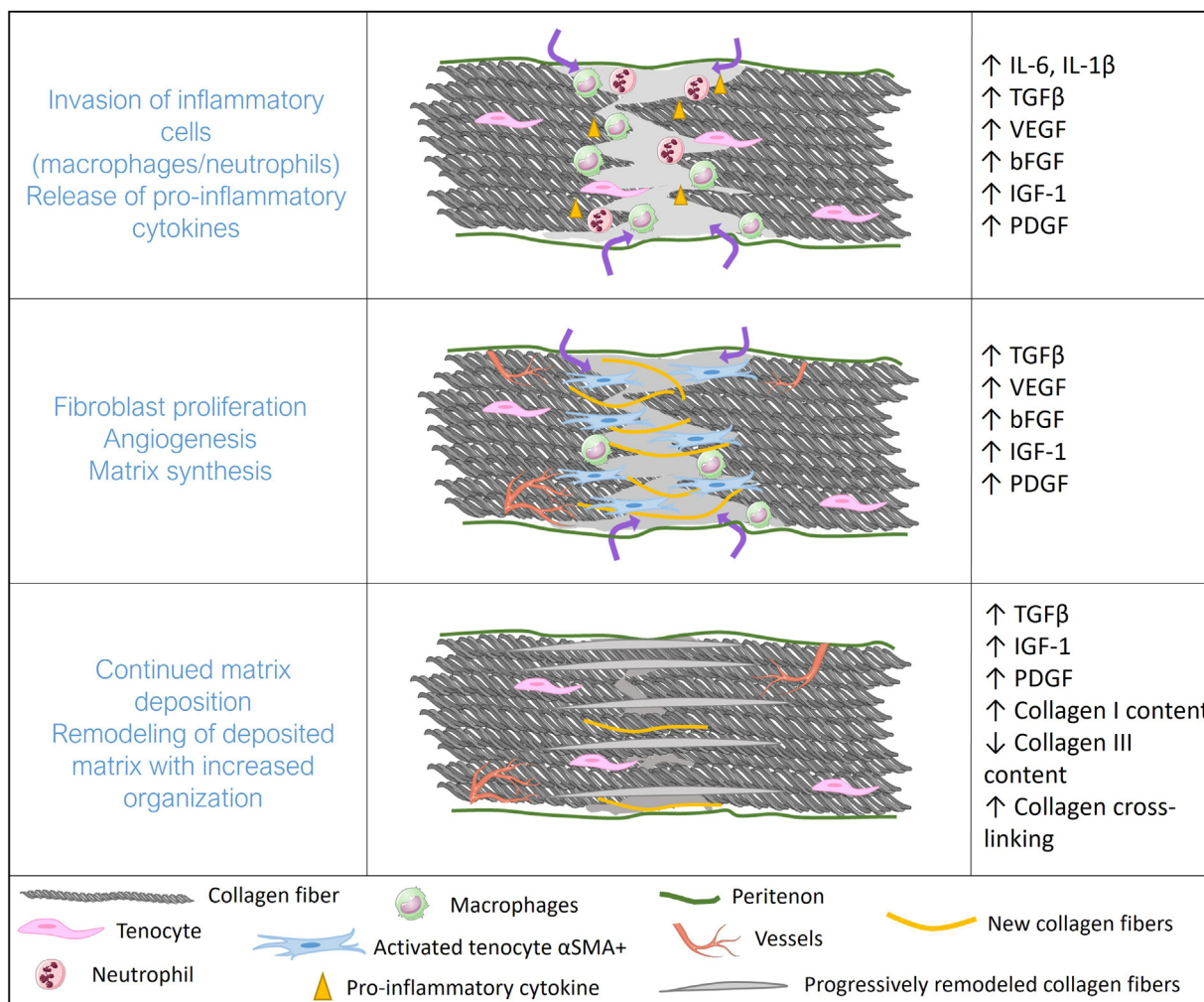


Fig. 1. Main stages of the healing process. The healing process is composed of 3 successive phases: an inflammatory phase then a proliferative phase and finally, a remodeling phase.

to gradual accumulation of matrix damage over time [71]. One of the major limitations of human studies on tendinopathy is that tendon biopsy samples are usually obtained when patients are sufficiently symptomatic to require surgery (tendon tear) and such material represent chronic, end-stage rather than early-stage disease. However, our current understanding of the pathogenesis of tendinopathy has evolved based on animal models of overuse tendinopathy and from tissue specimens from patients. Thus, early cellular and molecular alterations that contribute to the development of tendinopathy are becoming more widely understood. Emerging evidence indicates involvement of an inflammatory component in tendinopathy pathogenesis, with inflammatory cells and cytokines being the central regulators of tendon ECM remodeling [72].

Many key inflammatory interactions occur in the earlier stages of repetitive tendon microtrauma [73]. Initial trigger for inflammation could be minor trauma of tendon (mostly unrecognized) which activates innate immune system through DAMPs. ECM degradation products are potential DAMPs that upregulate pro-inflammatory mediators like IL-1β, TNF-α and IL-6 through NF-κB activation [74]. Increased expression of IL-17 has also been detected in human samples of early tendinopathy [71]. At these early stages, changes in tissue microenvironment and activation of the innate immune system interact at a crossroads between reparative versus degenerative “inflammatory” healing. Repetitive biomechanical stress and its associated damage play a key part in the immune system

response [75]. Array data from human tendinopathic tissues showed aberrant regulation of the IL-6 pathway (increased expression of IL6-R and activation of STAT3 (its intracellular regulator signal transducer and activator of transcription 3) [76,77]. In addition, recent findings coming from single-cell RNA-sequencing showed that different tenocyte subpopulations are present in normal and damaged tendons [78], and that cells driving healing originate from the epitenon [17]. These data suggested different differentiation trajectories of TSPC in normal versus diseased tendon. Thus, chronic inflammatory infiltration could sustain chondrogenesis/endochondral ossification [78] or deviation towards fibrous scarring [17], leading to inferior tissue quality. However, new studies will be needed in the future to further investigate the key cellular interactions involved in the development of tendinopathy to improve patient management, which remains a medical challenge to date.

Disclosure of interest

The authors declare that they have no competing interest.

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