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Defining the profile: Characterizing cytokines in tendon injury to improve clinical therapy

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ABSTRACT

Cytokine manipulation has been widely used to bolster innate healing mechanisms in an array of modern therapeutics. While other anatomical locations have a more definitive analysis of cytokine data, the tendon presents unique challenges to detection that make a complete portrayal of cytokine involvement during injury unattainable thus far. Without this knowledge, the advancement of tendon healing modalities is limited. In this review, we discuss what is known of the cytokine profile within the injured tendinous environment and the unique obstacles facing cytokine detection in the tendon while proposing possible solutions to these challenges. IL-1β, TNF-α, and IL-6 in particular have been identified as key cytokines for initiating tendon healing, but their function and temporal expression are still not well understood. Methods used for cytokine evaluation in the tendon including cell culture, tissue biopsy, and microdialysis have their strengths and limitations, but new methods and approaches are needed to further this research. We conclude that future study design for cytokine detection in the injured tendon should meet set criteria to achieve definitive characterization of cytokine expression to guide future therapeutics.

1. Introduction

Tendon injury is a prevalent musculoskeletal disorder experienced by approximately 32 million people within the United States each year.[1] Athletic animals, such as agility dogs and sport horses, also frequently experience tendon injuries.[2,3] Whether the injury is as severe as a complete rupture or an on-going chronic tendinopathy, tendons are naturally slow to heal and rarely repair themselves to a completely healthy state, resulting in significantly delayed return to training and competition for athletes.[4] Inadequate restoration of damaged tissue can result in a 23–67% re-injury rate of the affected tendon.[5]

Modern therapeutics seek to enhance and improve the efficacy of tendon healing via the manipulation of innate cellular components and their cytokine cross-talk with infiltrating immunomodulatory cells. While once thought of as limited to the realms of innate and acquired immunity, it is now known that cytokines also play a pivotal role in cellular repair, cellular genesis, proliferation, and apoptosis.[6]

Progress has been hindered due to a lack of complete understanding of the tendinous environment during injury regarding the timing and presence of cytokines relevant to the healing process. Cytokine detection studies have been conducted in attempts to answer outstanding questions, but challenges to study design prevent uniform conclusions from being drawn. To advance regenerative medicine for tendon injury, future research must seek to overcome these obstacles by utilizing *in vivo* temporal cytokine detection methods in species, anatomic, and pathology specific models.

2. Cytokines and tendon healing

Within the tendon, there are three distinct compartments in which cellular communication via cytokine signaling has proven to relate to tendon injury. According to Millar et al. the *immune sensing compartment* of the tendon is where resident immune cells, such as fibril-embedded mast cells and macrophages, patrol and respond to tissue damage. The *stromal compartment* involves the extracellular matrix (ECM) which houses tenoblasts, tenocytes, and tendon stem/progenitor cells (TPSCs). Both paracrine and autocrine signaling occurs here, as the cells self-regulate and contribute to tendon maintenance and repair. Finally, the *infiltrating compartment* concerns immune cells drawn into the tendon proper via paracrine signaling. These T-cells, mast cells, and macrophages represent both pro- and anti-inflammatory mediators during tendinopathic conditions. Together, the cellular components of each

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compartment maintain tendon homeostasis or initiate a coordinated pro-inflammatory environment for reparative purposes. Disrepair and chronic tendinopathy is proposed to occur when mediator dysregulation perpetuates signalment of the pro-inflammatory state, causing further degeneration of tendon tissue rather than appropriate reconstruction.[7]

2.1. The acute inflammatory cascade

A tendon will sustain significant damage when subjected to strain beyond its variable yield point. Once injured, a chronological sequence of specific events will occur to promote healing and repair of the tendon. These events occur rapidly after tendon failure, establishing a pro-inflammatory environment within 2–3 days post-injury.[8]

During early stages of tendon injury, pro-inflammatory cytokines are released to initiate the process of cellular excavation and degradation. Three vital mediators of this phase include interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6).[9] This trio of pro-inflammatory cytokines function synergistically to initiate internal and external cellular mechanisms during the inflammatory phase including the most overt clinical signs of tendon injury which are heat, swelling, and pain.

IL-1β is synthesized in response to injury or immunological challenge to serve as a mediator of cell proliferation, vasodilation, differentiation, and apoptosis.[6] In animal injury models, IL-1β gene expression was found to be elevated during the first ten days following tissue damage, then tapered in expression following the acute inflammatory phase.[10] In the tendon specifically, IL-1β is released not only by infiltrating neutrophils and macrophages, but initially by tenocytes as they become over-stretched or deformed by injury.[11] Cross-talk between damaged tenocytes and arriving immune cells induces cell surface receptor modification and upregulation of additional pro-inflammatory cytokines.[12] The result is downregulation of type-I collagen expression from tenocytes and increases in specific collagenases such as matrix metalloproteinase-1 (MMP-1), which degrades the ECM surrounding the damaged tissue.[13,14]

While pro-inflammatory, IL-1β also contributes to the resolution of the inflammatory state of the injured tendon. It directly causes the production of a specialized pro-resolving mediator coined lipoxin A-4 (LXA-4) to be released from the cytoplasmic processes of tenocytes. LXA-4 binds to the FPR2/ALX receptor on monocytes, M2 type macrophages, and tenocytes, invoking both paracrine and autocrine effects. LXA-4 inhibits neutrophilic infiltration and possibly induces M2 type macrophages to serve as an endogenous signal to halt inflammation.[15,16] *In vivo* examination of timing and correlation between the presence of IL-1β, FPR2/ALX upregulation on tenocytes, the accumulation of LXA-4 and its effects on macrophage phenotype differentiation is warranted to paint a more complete picture of the interplay that occurs between each event.

TNF-α is released as an early pro-inflammatory response to the pathological tendon environment, stimulating both catabolic and anabolic effects.[17] In animal injury models, TNF-α was found to be present as soon as 2 h post-insult to the tendinous tissue and remained elevated for at least nine days.[9] One of the earliest detectable cytokines following tendon injury, TNF-α is produced by disrupted tenocytes and is known to activate NF-κB signaling pathways which lead to the synthesis and release of lipoxygenase-2 (LOX-2), prostaglandin E2 (PGE2), cellular adhesion molecules, inducible nitric oxide synthase (iNOS), chemokines, and other pro-inflammatory cytokines like IL-1β and IL-6.[18,19] Together with IL-1β, TNF-α is a strong amplifier of MMP-1 and elastin while inhibiting type-I collagen production by tenocytes.[17] In addition to having autocrine and paracrine effects on tenocytes, mast cells, and macrophages, TNF-α modulates the surrounding vascular endothelial cells to promote vasodilation and also induces vascular endothelial growth factor (VEGF) for the promotion of neovascularization at the injury site.[11,20]

As previously mentioned, FPR2/ALX receptors can be found on the cytoplasmic arms of tenocytes throughout the inflammatory phase.[15] Cultured tenocytes, when exposed to 10 ng/mL and 100 ng/mL of TNF-α were found to reduce the length and morphological structure of those cytoplasmic processes.[17] Given the early timing of arrival of TNF-α, it may be that this effect on tenocytes acts as a physical modification to discourage the upregulation of LXA-4 until the environment is cleared of damaged tissue. There may be a direct correlation between the regression of TNF-α and the expansion of tenocytes' FPR2/ALX receptors. The potential connection between the two events may be linked to the resolution of the inflammatory phase and the beginning of the proliferative phase. Further *in vivo* research is warranted into discovering if such a link exists.

IL-6 is the final major pro-inflammatory cytokine that has a significant presence in the inflammatory phase. It is pleiotropic, a regulator of both humoral and cellular responses, and can be found within both tendinopathic tissues as well as healthy tendons that experience strenuous, prolonged exercise.[9] In the early stages following tendinous injury, the pro-inflammatory cytokines IL-1β, TNF-α, and direct contact with activated T-cells influences tenocytes to upregulate IL-6 expression via autocrine and paracrine amplification loops.[19,21] IL-6 is a strong proponent of hematopoiesis and neovascularization to the damaged site via VEGF dependent angiogenesis via the STAT3 pathway activation.[22] It also increases vascular permeability, assisting with the infiltration of $CD4^+$ and $CD8^+$ T-cells drawn to the site from surrounding lymphatics.[19,23]

IL-6 serves as an early-stage promoter of inflammation but persists in the proliferative environment after other pro-inflammatory mediators diminish. IL-6 has proven to act as an anti-inflammatory regulator in cultured monocytes, blocking the upregulation and release of TNF-α.[24] Also, while IL-1β and TNF-α upregulate MMPs to debride and degrade the tendon ECM, *in vivo* microdialysis research has shown that IL-6 can have a positive effect as a growth factor for type-I collagen under set conditions.[25] This coincides with the delayed healing response and inferior mechanical ECM construction and fibril organization seen in IL-6 knock-out mice.[26] IL-6 has also shown a strong proliferative capability towards tendon stem/progenitor cells (TSPCs), once again suggesting that it may play an important role in tendon healing.[22]

2.2. Proliferative phase cytokines (2–6 weeks post injury)

The resolution of the inflammatory phase and initiation of healing begins roughly two weeks post-tendinous injury. In human Achilles tendon ruptures, the core pro-inflammatory mediators IL-1β and TNF-α were markedly decreased or absent by fourteen days and levels of IL-6, IL-8 and IL-10 increased.[24] Given that IL-6 has dual properties, this general upregulation of anti-inflammatory cytokines was credited for causing the sudden rise in reparative effects seen within the damaged tendon at this time point.

M2 macrophages expand in number during this phase and have been shown to release varying combinations of IL-10, IL-4, and IL-6 when stimulated *in vitro*.[27] The influx of these cytokines stops the creation of pro-inflammatory cytokines, inhibits the production of ECM degrading MMPs, induces the apoptosis of neutrophils, differentiates and increases proliferation of tenocytes, and upregulates collagen production.[28] The cumulation of these effects is evident as granulation tissue is constructed in areas of tendon damage within the ECM.[29] By tracing collagen production patterns, this reconstruction begins in the outer layers of the tendon before moving inward towards the thicker endotendon. The more internally located tenocytes lay down larger, more mature molecules of collagen for the tendon core, re-stabilizing the tendon infrastructure. A strong response coordinated by M2 macrophages, activated T-cells, and these tenocytes results in intrinsic healing for the tendon by maintaining gliding capability and preventing adhesion formation, although the restoration of the tendon is incomplete. The anti-inflammatory milieu of cytokines predominant in this phase stimulate tenocytes to produce a much greater amount of type-III collagen

rather than the sturdy, primary type-I collagen found in healthy tendons.[21,30] This change in composition requires an additional phase to restore the tendon to its pre-injury confirmation.

Chronic tendon injuries that do not progress past the inflammatory period into the subsequent healing phases tend to have a persistent macrophage response and NF-κB signaling.[31,32] It is likely that soluble factors from the local tenocytes play an important role in switching the macrophage response from an M1 to an M2 biased response, although it's not well understood which cytokines are most important for the shift in phenotype and why this shift doesn't occur in some chronic injuries.[33]

2.3. Remodeling phase cytokines (6 weeks-2 years post injury)

In the final phase of tendon repair, the haphazard collagen type-III repair performed by tenocytes is replaced with a more durable, collagen type-I constructed ECM. This matrix replaces the disorganized proliferative phase scaffolding with parallel orientated fibrils cemented into place with extensive covalent cross-linking.[28] Several growth factors contribute to this reformation: bone morphogenic proteins (BMPs), transforming growth factor beta (TGF-β), and insulin-like growth factor 1 (IGF-1).[34]

Despite what the name implies, BMPs do not strictly act upon bone formation. BMP signaling also plays a role in tendon remodeling.[35] In an Achilles tendon repair study, BMP12 was shown to cause elastin and collagen type-I expression in rat tenocytes, resulting in an increase of fibril diameter and better organization within core lesions.[36] BMP12 has also been found to reduce adhesion formation and fibrosis in a rat Achilles tendon transection repair model.[37] Additionally, Li et al. found that exogenous BMP2 application enhanced tenocyte migration using a rat patellar injury model. Histochemistry results of this *in vivo* study revealed that the BMP2-treated tendons promoted tenocyte migration to the injured tendon's core and exhibited more complete repairs than controls by Day 40 post-application.[38] In addition to tenocyte stimulation, *in vitro* and *in vivo* studies have proven that BMPs also stimulate multipotent TSPCs to differentiate and contribute to tendon healing.[39]

All three TGF-β isoforms exist within the tendon healing environment and significantly increase the production of type-I collagen, fibronectins, and glycosaminoglycans.[34] TGF-β serves as a powerful anti-inflammatory component during the remodeling phase, acting on tenocytes and macrophages to inhibit the production of TNF-α, IL-1β, and IL-6.[40] However, a delicate balance must be reached regarding TGF-β. In early studies experimenting with exogenous TGF-β therapy, an overabundance of TGF-β resulted in tendon fibrosis and scar tissue.[41] Conversely, in an early study using rabbit models, antibodies targeting TGF-β1 caused an increase in flexion and mobility for tendon repair models, while TGF-β1 + TGF-β2 simultaneous targeting resulted in average healing.[42]

IGF-1 has a stimulatory effect throughout the tendinous environment, working on cartilage and the plethora of cellular components within the tendon proper. This anti-inflammatory cytokine dictates tenocyte migration to areas in need of reconstruction, induces tenocyte division and proliferation, expression of matrix components, and

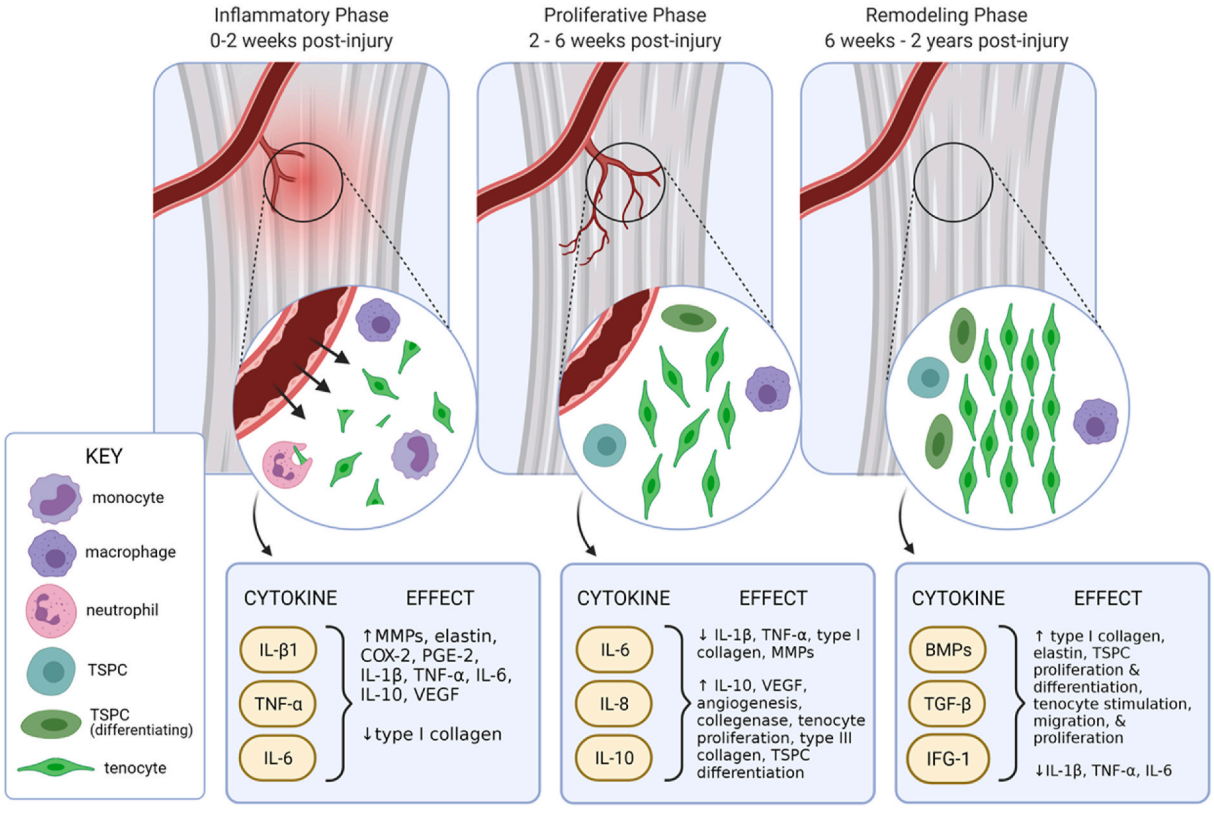

Fig. 1. Cytokine signaling during the primary phases of tendon repair. During the initial two weeks post-injury, a pro-inflammatory environment is established through the release of IL-1β, TNF-α, and IL-6 by damaged tenocytes and infiltrating immune cells. These pro-inflammatory mediators collectively promote extracellular matrix degradation, neovascularization, vascular permeability, elastin production, and have anti-apoptotic effects on healthy tenocytes. Increased expression of IL-6 at the end of the acute inflammatory phase suppresses the production of other pro-inflammatory mediators in the tendinous environment and initiates the proliferative phase of repair. During the proliferative phase, naïve macrophages differentiate to a M2 phenotype and produce anti-inflammatory cytokines including IL-4, IL-8, and IL-10. This shift in the cytokine milieu slows the phagocytic removal of damaged tissues, induces proliferation of tendon stem/progenitor cells, and increases type-III collagen fibril formation for initial reconstruction of the extracellular matrix. Several weeks after initial injury, the remodeling phase and its associated growth factors take precedence in the tendinous environment. Elevated levels of TGF-β inhibit production of IL-1β, TNF-α, and IL-6, BMP's stimulate tenocytes to increase production of type I collagen fibrils and elastin to promote a more organized, permanent reconstruction of the tendon, and IGF-1 regulates proliferation and migration of tenocytes.

contributes to the upregulation of type-I collagen expression.[34] IGF-1, when applied simultaneously with BMP12, appears to enhance tendon healing via an improved rate of collagen synthesis.[41] In a four-year study of tendinopathy cases conducted on naturally occurring superficial digital flexor tendon (SDFT) damage in Thoroughbred racehorses, intralesional application of IGF-1 was shown to lessen the severity of core lesions.[43] The anabolic growth factor also works synergistically with platelet-derived growth factor (PDGF), as studies have shown that maximum tenocyte proliferation occurs when both are introduced into the injured tendinous environment in comparison with IGF-1 application alone.[44] Cytokine expression during each stage of tendon healing is reviewed in Fig. 1.

2.4. Cytokine detection and its applications

In addition to conveying an alert of cellular damage, needed maintenance or repair, cytokines can also serve as markers of cellular involvement. By their presence or absence, researchers can evaluate the concentration and specificity of these signals to assess the status of the tendon. Currently, studies have been conducted to capture cytokine cross-talk in efforts to evaluate standard levels for what constitutes a "normal" or healthy tendon, and conversely, which cytokines indicate a pathological condition.[6]

Furthermore, detecting the precise timing regarding when specific cytokines arrive following injury is crucial to developing effective treatment. Because the bulk of the tendon has a slow cellular turn-over rate, the healing process takes longer to complete than in most other areas of the body. It can take upwards of 9–18 months for the equine SDFT to return to normal function when assisted by aggressive therapeutic intervention.[45] Retrospective studies focusing on human athletic injury have also reported significantly higher return to training times and reinjury of intratendinous tears when >50% of the cross-sectional area of the affected tendons become compromised.[4]

Conservative management of tendon injuries has included non-steroidal anti-inflammatory drugs (NSAIDs) to relieve pain and swelling, but NSAIDs can inhibit tendon remodeling and healing in animal models through the suppression of prostaglandin production, particularly if given during the acute stages of injury.[46–48] One area of active research is the use of biologics to manipulate the cytokine microenvironment within an injured tendon to activate innate and potential exogenous healing mechanisms thereby promoting more efficient, successful healing. Mesenchymal stem cells (MSCs) constitutively secrete a variety of immunomodulatory trophic factors and the MSC secretome can be further enhanced by licensing with pro-inflammatory cytokines.[49] MSCs promote proliferation and regeneration of tendon tissue when used *in vitro* under specific conditions; however, the temporal expression of cytokines during tendon healing *in vivo* and how those cytokines influence the MSC secretome is still not fully understood. Future research is warranted to further explore how and when MSC application should be utilized to optimize cytokine expression and healing.

3. Study design & methods of collection

Among the studies that have been conducted regarding the assessment of cytokine involvement of tendon injury, there are three primary study designs: cell culture, tissue biopsy, and microdialysis. Each has its advantages and disadvantages in exploring the cytokine environment and which model is appropriate depends on the mechanism being investigated, the type of injury, or whether the goal is to measure normal or pathologic cytokine responses.

3.1. Cell culture

In vitro tendon research aimed at the examination of individual cellular component's contributions to the overall environment and cytokine population will often make use of cellular culture methods. Two cell types are most often found in use—tenocytes and TSPCs. Since these cells form most of the tendon's cellular composition and are the primary targets for cytokine signaling within tendon injury, numerous studies featuring these cells have been conducted over the years. Cultured tenocytes and TPSCs can be used to investigate biomechanical stimulating factors, interplay between infiltrating and resident cellular populations, and the direct effects of pro- and anti-inflammatory cytokines upon the most basic elements of tendon structure.

Researchers have examined the effects of exercise on tenocytes in attempts to determine the cellular origins of chronic tendinopathy. *In vitro* studies using culture models conclude that cyclic mechanical stretching of tenocytes causes morphological and physiological changes to the cells. In early studies, Wang et al. used cultured human patellar tenocytes obtained from patients undergoing reconstructive surgery to investigate the downstream effects of COX-1 and COX-2 in relation to PGE2 production by the cells. Following *in vitro* expansion of tenocytes, the cultures were subjected to 24 h of cyclic mechanical stretching at varying magnitudes to mimic the repetitive effects of exercise on the tendon. From this study, it was determined that PGE2 production in the tendon was magnitude dependent and levels showed an interdependence between the enzymes and PGE2 synthesis by tenocytes.[50]

Once the basic cytokine profile of mechanical stretching was established, pro-inflammatory cytokines and their effects on tenocytes undergoing manipulation were investigated. One study conducted by Yang et al., in 2005 focused on IL-1β and the signaling interplay that occurred after its introduction to tenocytes that had undergone 4 h of cyclic uniaxial stretching in silicone culture dishes. COX-2, MMP-1, and PGE2 levels of expression were evaluated via RT-PCR and enzyme-linked immunoassay (ELISA) in both control and experimental groups. This study concluded that the presence of IL-1β caused stressed tenocytes to upregulate their expression of pro-inflammatory markers, proving that increased strain could have deleterious effects on tendon infrastructure.[51]

The main advantage of using cell culture to study the tendon is also this type of study's main disadvantage—the primary cellular components are the sole focus. This can be beneficial when examining the most basic interactions that occur within the tendinous environment; however, tenocytes or TSPCs are not the sole players on the tendon stage. Therefore, cell cultures are limited in their narrow representation. Al-Sadi et al. recognized this design flaw, and in 2011 conducted a study with the intent on recreating the tendon in a more authentic manner. Peripheral blood mononuclear cells (PBMCs) were introduced to isolated rabbit tenocytes to develop a co-culture that was more representative of an *in vivo* tendinous environment, leveling more accuracy to their conclusions. They were able to surmise that neutrophils suppressed type-I collagen production and stimulated TNF-α synthesis. They also found that the leukocytes significantly increased levels of MMP expression by the tenocytes, connecting ECM degradation with the presence of inflammatory cells.[19] Given that this study was conducted in a time when tendinopathy was considered to be void of inflammation, the results challenged what was known regarding inflammatory cell involvement within the pathological tendon. More recent studies incorporated similar indirect transwell co-culture design methods to further investigate time dependent effects of TNF-α on gene expression and tenocyte complement regulation.[52]

Despite its inherent flaws, cell culture remains a valuable model of study design for tendon research, particularly when human participants are involved. Any physical interference with tendon tissue, whether by collecting a biopsy sample or via insertion of a microdialysis catheter, causes disruption in an area that has been proven to heal poorly and insufficiently. While in pathological studies this complication can be circumnavigated by coinciding collection with necessary surgical repair, obtaining human tendon tissue samples and data from the normal tendon has considerable ramifications. For ethical purposes, cell collection offers a valid solution in that one sample can be multiplied

and repurposed as often as necessary.

3.2. Tendon biopsy

The biopsy model has proven to be an effective holistic method of cytokine detection for tendon studies and has been used more extensively in tendon research to date than microdialysis. Direct tissue collection from healthy and pathological tendons allows for an *in vitro* examination of collective cellular components and the presence of cytokine genetic markers within the tissue on a more inclusive scale than that of cell culture.

One of the first studies to examine histological immunodetection of cytokine markers within healthy and diseased tendons was performed in 2002 by Hosaka et al. In the study, biopsies were taken from healthy and inflamed SDFTs from Thoroughbred racehorses and IL-1β, TNF-α, and interferon gamma (IFN-γ) were measured via immunohistochemistry. This biopsy study was able to map cytokines to specific locations within the tendon, tracing the strongest concentrations of the pro-inflammatory cytokines to tenocytes within the endotendon of the diseased SDFTs.[53] While they were able to detect a cytokine presence in their tendinopathic cases and calculate distribution percentiles of the observed populations, as with most biopsy studies, the results were limited in scope regarding the temporal expression of cytokines.

In 2007, Berglund et al. used a rabbit model to detect cytokine presence in surgically repaired flexor tendons and their related tendon sheaths at set time stamps of 3, 6, 12, and 24 days after injury. By using RT-PCR analysis, the researchers were able to capture gene expression of IL-1β, COX-2, TNF-α, MMP-13, and iNOS throughout the different phases of tendon healing. Interestingly, they were also able to demonstrate that gene expression of IL-1β, COX-2, and TIMP-1 were upregulated within the tendon sheath, suggesting an interrelationship during healing in the two tissues.[54] However, this study did not include the remodeling phase of tendon healing and RNA expression may not be the most accurate method in detecting cytokine levels as there is post-transcriptional regulation of protein synthesis.

Surgical repair biopsy models paired with RT-PCR analysis, despite its potential detection flaws, have been a common choice for study design for tendon research. A 2009 study performed by Loiselle et al. analyzed MMPs and their effects on type-III collagen production in flexor digitorum longus (FDL) tendon repairs in mice. Multiple MMPs were analyzed and the formation of scar tissue was monitored for 35 days. Biopsies were viewed histologically for improved organization and fibril reconstruction. At nine weeks, repaired tendons in the remaining mice were subjected to biomechanical evaluation via applied tension and force-displacement data was collected.[55] The addition of visual and tactile evaluation to this detection study improved its quality, but it was limited in scope by focusing only on cytokine involvement of collagen upregulation.

While animal models are more commonly used for evaluation, human biopsy studies have been performed. Cadaver tendons, used in studies such as Legerlotz et al., in 2012, were acquired for analysis of the normal Achilles tendon. These tendons were used in comparison with samples obtained during surgical repair of patients with diagnosed chronic Achilles tendinopathy or ruptured Achilles tendons. The processed tissue samples were evaluated by RT-PCR to reveal the genetic presence of COX-2, IL-6, and VEGF. This study incorporated the use of cadaveric tendons as a baseline reference of the normal tendon environment.[56] But, given that these samples were taken from a metabolically inactive state, from individuals who may have been suffering from undiagnosed tendinopathies, creates a degree of unreliability for this study and for human-based tendon research. The on-going ethical issue with sampling from normal human tendons creates a stronger argument for the use of animal models in this field.

More recently, human tendon biopsy studies have shifted towards comparing chronic to acute stage tendinopathies. In addition to the baseline usage of cadaver tendons, Klatte-Schulz et al. formatted their study around this approach to investigate chronic tendinopathies, chronic ruptures, and acute ruptures in a human model. Immunoassays were able to detect inflammatory cell infiltration in all but the intact cadaver tendons. RT-PCR indicated MMP presence in both chronic tendinopathies and chronic rupture samples and also detected IL-1β, TNF-α, COX-2, and IL-6 in all three pathological tendons.[57] This study design was able to shed light on the cytokine environment of human tendons undergoing pathological changes and rupture without completely relying on the use of cadavers for sole comparison; however, real time collection involving temporal plot points of cytokine detection was not accomplished by this study and cannot be adequately achieved via any biopsy study. This hindrance has led some researchers to use microdialysis as a measure of detection in favor of the more traditional biopsy methods.

3.3. Microdialysis

Unlike cell culture or tissue biopsy, microdialysis collection is a method that allows for an *in vivo* examination of metabolic changes and protein expression. In application with tendon studies, a microdialysis catheter is inserted into the peritendinous space. Then, sterile aqueous solution appropriately formulated to match the ionic composition of the area is pumped into the catheter, retrieved, and measured. By calculating the concentrations of the perfusate against the collected dialysate, targeted analytes such as cytokines can be determined in a temporal fashion.

Langberg et al. performed some of the earliest studies making use of microdialysis to explore the effects of cytokines on the tendinous environment. Both of the following two studies used human exercise models to gather concentration levels of pro-inflammatory markers within the peritendinous space adjacent to Achilles tendons.[58]

The earlier of the two studies compared levels of PGE2 via radioimmunoassay collected both at rest and during repetitive mechanical loading of six participants possessing normal, healthy tendons. Precautions were taken to eliminate effects of insertional trauma from the data set by allotting a subsequent period of recovery prior to starting collection of dialysate for analysis. PGE2 was shown to have a high recovery rate during exercise and at rest.[58] Since previous cell culture and tissue biopsy studies had already established that PGE2 levels become increased after exercise, the results of this study established microdialysis as an effective detection method to make use of for future tendon research.

The second tendon-based microdialysis study performed by the Langberg research team explored IL-6 interstitial concentrations at allotted time points before and four days after a prolonged running event. 3000 kDa microdialysis catheters were perfused with ringer acetate solution containing type-IV collagen 2 h prior to exercise and then regularly at 2, 24, 48, 72, and 96 h following activity. The results indicated a sharp increase in IL-6 production corresponding to the increase in mechanical stimulation, with a steady decline returning to normal levels shortly after 72 h. This study validated microdialysis collection for the use of detecting interleukins, as the results matched those found in previous biopsy evaluations. It also created discussion regarding tenocytes and their role in IL-6 synthesis during tendon stress.[59]

While researchers became more cognizant of the potential for microdialysis use in tendon study, increased scrutiny was also applied. As discussed in the Langberg cases, a recovery period had been incorporated into the study design to offset any induced trauma caused by catheter implantation. Still, the question remained—how long of a rest period post-insertion is sufficient to obtain data void of iatrogenic effects?

In 2007, Olesen et al. aimed to answer this question by examining the effects of microdialysis catheter insertion on IGF-1, its binding proteins (IGFBP) and the type-I collagen synthesis marker, procollagen I COOH-terminal propeptide (PICP). Two catheters were implanted diagonally

and ventrally along the Achilles tendon in a control group who did not engage in strenuous exercise and in participants who performed a 36 km run. Total catheter insertion events occurred six times over the course of the study, with samples collected from both cohorts at allocated time points over a period of five days. The results of their efforts indicated that control groups also exhibited increased levels of both IGF-1 and PICP, thus implying an iatrogenic complication to microdialysis as a detection method. Although other common pro-inflammatory cytokines were not evaluated, it was the first published study to explore the effects of catheter insertion on collection data. Recommendations were made by the authors that future tendon microdialysis experiments compensate for trauma by minimizing the number of insertions in attempts to offset data error.[60]

Future research was slow to take heed of the warning, prompting commentary and further discussion regarding the issue.[61] In 2012, Ackerman et al. investigated the cytokine profile of surgically repaired acute Achilles tendon ruptures two weeks post-operation. The study design gave respect to the deleterious consequences on multiple probing, remodeling itself so that microdialysis catheter implantation was performed in a single event. In each patient, one catheter was placed in the peritendinous space on the lateral distal aspect of the surgically repaired Achilles tendon and another was positioned similarly on the contralateral side. Once inserted, a preformulated perfusate was administered and the returning dialysate was used to assess the concentration of pro- and anti-inflammatory cytokines. PGE2 was assessed via ELISA, while IL-1β, TNF-α, IL-6, IL-8, and IL-10 were evaluated via a bead-based immunoassay and flow cytometry. A further precaution in addition to single insertion was taken to ensure accuracy; the first sampling of the four dialysates collected over the course of 2 h was discarded. The results from the nine patients of the study revealed a steady anti-inflammatory state, as expected during the beginning of the proliferative phase, with no detectable traces of PGE2, IL-1β, TNF-α appearing in the dialysate. In contrast, levels of the anti-inflammatory cytokines IL-6, IL-8, and IL-10 were markedly upregulated.[24]

This study provided an *in vivo* look at the cytokine environment following surgical repair of an Achilles tendon. But, while the study was adapted to eliminate error due to insertional trauma, the study limited itself to a single time point of investigation. To gain a more temporal evaluation of cytokine dynamics within the pathological tendon, studies utilizing single insertion events could be used consecutively during each of the phases of the inflammatory cascade, allowing ample time to offset any trauma. To be even more accurate in cytokine assessment, it may be necessary to abandon microdialysis as a collection method altogether. Current research is investigating the use of ultrafiltration as a microdialysis substitute for *in vivo*, long term cytokine detection. Probes utilized in this method are designed for long term implantation, allowing a single insertion event to last for the duration of membrane-based sampling studies. Exploration of this technique is warranted to create a true portrait of cytokine interplay during the length of tendon healing for further application towards therapeutic developments.[62–64]

4. Discussion

The challenges to cytokine detection in the tendon are numerous and must be addressed to advance our understanding of the pathophysiology of tendon healing. Without such investigation, portrayal of biological components is incomplete. Most importantly, without fully comprehending the molecular signaling that occurs during injury, researchers are blind to the pathways directing cellular repair and manipulation without consideration to cellular cross-talk can result in unintended consequences. As a result, therapeutic applications are stymied, and most have proven ineffective in drastically reducing the tendon re-injury rate. Future research endeavors should build upon the knowledge that has already been accumulated in the aforementioned studies, while avoiding inherent flaws and considering the following when developing study design.

While some parallels can be drawn across species, in the case of tendon-based research it remains evident that all model organisms have unique features when compared to humans and the pan-species equivalence hinders progress in the field. While each species' tendons are composed of similar cellular components and healing mechanisms for the purpose of creating healing modalities, the model organism should be carefully chosen. Researchers must consider when investigating mechanisms of tendon healing whether the cytokine environment found in the tendons of a model organism is truly representative for their purposes and future applications. For example, murine tendons are not subject to the load experienced by tendons of large animals, so that may create disparity in the cytokine landscape between the two.[65] Gene expression of inflammatory mediators, transcription factors, and matrix remodeling proteinases relevant to healing in tendinopathic conditions varies amongst species; however, close scrutiny has revealed that equine and ovine tenocytes exposed to inflammatory conditions produce more comparable expression levels to human-derived tenocytes than mouse or rat tenocytes.[66] Therefore, if a study wishes to evaluate cytokines to create a more effective reparative therapeutic for use in a human model, it is logical to use a large animal model to more effectively replicate the impact of tendon loading on cytokine responses and gene expression.

As a significant portion of tendon research is aimed at curing human tendinopathy and promoting repair, drawing conclusions for human application from murine or rabbit studies may create inaccuracies. *In vivo* human experimentation is limited by ethical restraint and is generally avoided. This drawback could be overcome by using equine models as the standard for tendon regenerative research for the study of Achilles tendinopathy, as the equine SDFT has been deemed a functional and clinical equivalent structure based on action, composition, use, and injury rate.[67,68] Importantly, unlike other large animals such as the sheep or pig, horses as athletes suffer from naturally occurring tendon injuries and undergo similar treatment and rehabilitation protocols as humans do with similar return to performance demands. However, there is a difference seen in the pathophysiology between the two species to be noted. Most human Achilles tendon injuries involve insertional sites, whereas horses suffer most frequently from core lesions. This discrepancy could be attributed to the dissimilarity between force distribution between the two species and should be taken into consideration. The proposed solution of using an equine model is not a perfect one; however, in the absence of human subjects, the horse is a functional substitute.

This concept of comparative test subjects also introduces the idea of equivalence between tendons themselves, that conclusions from a study examining the cytokine environment displayed in a supraspinatus tendon should be inherently considered relevant to an extensor tendon. Degenerative change often occurs prior to tendon rupture and those alterations are more likely to be associated with high functioning, energy storing tendons.[3] This indicates that an increased cytokine presence may naturally occur in more load-bearing tendons. Given that the cellular activity and biomechanics of flexors, extensors, positional, and energy storing tendons all vary to a degree, can accurate conclusions be blanketed across the entire tendon spectrum in terms of their cytokine involvement during injury and repair? Assumptions should remain guarded until repeated studies are conducted on each variety of tendon, as the variability in targets assessed creates another element yet to be ameliorated. To promote greater efficacy of therapeutics, study designs should involve the examination of cytokine assessment within their intended recipient tendon to avoid potential inaccuracy.

Additionally, there are significant degrees of variability regarding pathophysiology in tendon studies that should be taken into consideration when drawing parallel conclusions. Early research was initially conducted with the exercise model, exploring the relations of mechanical overuse with tendinopathy. These studies complicated broad interpretation by introducing variables of strict immobilization, prone recovery states, or even free exercise for their test subjects following exercise. Later investigation progressed into acute or chronic

tendinopathies and rupture repair models, either natural in origin or surgically induced. More recently, 3D scaffolding has been developed to realistically mimic the tendon microenvironment from a pathophysiological perspective. These cellular models are mechanically stimulated via custom bioreactors to deliver appropriate levels of strain and compression for the purpose of measuring tenogenic differentiation of human-sourced bone marrow derived mesenchymal stem cells (hBM-MSCs) and their consequential gene expression of cytokine markers.[69]

The development of the 3D biological model allows for potential recreation of a variety of pathological scenarios, an exciting development as the only overwhelming generality discovered by this collective research is that one pathological state does not necessarily represent all. For example, IL-6 gene and protein expression has been found to be elevated in human Achilles and rotator cuff surgical repairs, but not in Achilles or rotator cuff tendinopathic cases.[9] Furthermore, it was concluded by the same systemic review that IL-1β, IL-6, IL-10, and TNF-α all differed in expression between tendinopathy, repair, and exercise studies. Evaluation of inflammatory cells and their associated cytokines varies among pathological states, even between acute versus chronic cases of tendinopathy.[70] Therefore, it is crucial for cytokine collection study design to reflect the pathological environment of its proposed therapeutic target.

5. Conclusions

For advancement of treatment, the ideal study design for cytokine detection includes species-specific test models, targeted tendons, and the pathological state aimed for therapeutic benefit. If possible, *in vivo* collection of cytokines should be performed, bearing in mind the effects of insertional trauma on the results of the study. Finally, to gather a complete assessment of the inflammatory cascade, ideal studies would examine the complete cytokine environment in each of the phases of tendon injury: inflammatory, proliferative, and remodeling. For maximum efficiency of therapeutic progress in this field of study, adhering to these parameters ensures the collection of data that accurately reflects cytokine activity within the injured tendinous environment.

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