Mesenchymal stem cell applications to tendon healing

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Summary

Tendons are often subject to age related degenerative changes that coincide with a diminished regenerative capacity. Torn tendons often heal by forming scar tissue that is structurally weaker than healthy native tendon tissue, predisposing to mechanical failure. There is increasing interest in providing biological stimuli to increase the tendon reparative response. Stem cells in particular are an exciting and promising prospect as they have the potential to provide appropriate cellular signals to encourage neotendon formation during repair rather than scar tissue. Currently, a number of issues need to be investigated further before it can be determined whether stem cells are an effective and safe therapeutic option for encouraging tendon repair. This review explores the *in-vitro* and *in-vivo* evidence assessing the effect of stem cells on tendon healing, as well as the potential clinical applications.

Key words: tendon, rotator cuff, stem cell, regeneration, cytokines.

Introduction

Failure of tendon regeneration following degeneration and damage has encouraged the search for biological, mechanical and surgical therapies to improve the strength and structure of healing tendons. The aetiology of tendon failure is multi-factorial. Mechanical factors have been shown to contribute to tendon tears. Once the tendon body is stretched beyond its elastic threshold, failure may ensue with inflammation of the tendon sheath and/or tendon degeneration[1]. Tendon damage can also occur when microtrauma forces are applied within the tendon's physiological threshold but the normal reparative mechanisms are overwhelmed[1]. Many tendon tears are prone to heal via scar tissue formation at the tendon-bone interface, resulting in regenerated tissue is usually weaker and more prone to failure[2]. Preceding degenerative changes play a key role in the pathogenesis of tears, as degenerative tendons are more likely to rupture than normal tendons during physiological loading, and have a reduced reparative potential[3,4]. Using rotator cuff tendons as a prime example, the incidence of tears increases with age and Nobuhara et al*.,* examined a series of tendon biopsies from patients aged 45 years and above, and reported that 81% of patients showed degeneration[5].

There is increasing interest in the application of stem cells to enhance tendon healing. It is proposed that self-renewing stem cells have the potential to recapitulate the embryonic tendon developmental signaling milieu. Ultimately this is thought to facilitate the regeneration of healthy functional tendon tissue rather than the weak scar tissue which normally forms during the tendon reparative response seen in adults following any form of damage or degeneration[6].

Despite some promising research, many questions regarding stem cells remain unanswered, such as its efficacy and safety. This manuscript will examine current literature regarding the application of stem cells to tendon healing in animals and in a clinical setting. Different modalities for enhancing the efficacy of stem cell healing will also be explored, such as growth factor, mechanical and genetic modulation.

Types of stem cells

Stem cells can simply be defined as a cellular population with the ability to self-replicate through mitosis to form daughter cell lines, which have the potential to terminally differentiate into a number of different cell lineages[7]. A number of sources exist for obtaining stem cells and thus stem cells can be classified based upon their tissue of origin. The most common stem cell sources are embryonic, perinatal (from the umbilical cord or amniotic tissue) or somatic adult cells. A more novel source of stem cells are induced pluripotent stem cells (IPSCs) which are initially mature adult cells that have undergone *in-vitro* modulation and obtained the characteristics of adult stem cells, such as pluripotency[8].

The majority of orthopaedic related stem cell research to date has focused upon adult stem cells rather than embryonic or perinatal stem cells, as the latter are associated with numerous regulatory and ethical constraints. IPSCs are a relatively new field that has generated a great deal of interest. Adult stem cells are predominantly either mesenchymal stem cells or haematopoietic stem cells. Dis-

tinction between these different stem cell populations is based upon their surface markers, although some overlap has been reported[9].

Mesenchymal stem cells (MSCs, which are sometimes also referred to as mesenchymal stromal cells) are defined by three specific characteristics. Firstly they are multipotent cells that are capable of differentiating into a number of daughter cell lines such as chondrocytes, osteocytes and adipocytes[10]. Secondly, MSCs are able to adhere to plastic. Thirdly, they present stem cell specific antigens on their surface. To date, no single stem cell specific marker has been identified, although panels of stem cell positive have been reported such as CD 31, 34, 40, 49c, 53, 74, 90, 106, 133, 144, 163, cKit and Slams[11]. The majority of such surface markers are associated with stem cells but are not unique to stem cells. Importantly, stem cells must display an absence of 'negative markers' that are used to identify other cell lineages, such as haematopoeitic or endothelial cells, such as CD 14, 31, 34 and 45[12,13].

The majority of MSCs utilized for orthopaedic applications are obtained from bone marrow tissue as these cells are relatively easy to access and provide relatively high numbers of MSCs compared to other sources. The iliac crest is the most common site for MSC harvesting, although a number of other sources have been identified. MSCs can be successfully aspirated while reaming long bones using a reamer-irrigation-aspirator (RIA), with comparable differentiation potential to iliac crest derived MSCs and superior numbers of total cells and colony forming units[14].

It is important to note that bone marrow aspirates provide very low yields of actual MSCs, which are estimated to account for only 0.001-0.01% of all nucleated cells[15]. MSCs can also be derived from other sources such as accessible adipose tissue which can also be relatively easily accessible, although these cells have an apparently reduced ability to differentiate into osteocytes and chondrocytes compared to bone marrow derived MSCs[16]. A number of alternative sources of stem cells have been identified, such as muscle, tendon, cartilage, synovium, blood, skin, testes, hair and scalp tissue although these are less commonly utilized[17-19].

Tendon progenitor stem cells

A tendon progenitor stem cell (TPSC) population has been identified in both humans and mice, with a greater incidence in tendon microenvironments or 'niches' containing tendon related growth factors such as fibromodulin and biglycan[18]. TPSCs appear to be a distinct population compared to tenocytes, and can be differentiated by the presence of stem cell markers such as tenomodulin, Oct-4 and SSEA-4[20]. In addition to differentiating into tenocytes, the TPSC population can differentiate into osteocytes, chondrocytes and adipocytes. A decrease in TPSCs with increasing age has been reported and may contribute to the increase in rotator cuff tears and reduction in healing potential noted with increasing age[21]. Culturing human tendon stem cells was enhanced when cultured on highly aligned nanofibers rather than randomly aligned nanofibers[22].

It has been proposed that delivering TPSCs to the sites of tendon healing may stimulate further tendon regeneration and healing. Deriving stem cells from tendon 'niches' may be important for encouraging differentiation into tenocytes rather than fibroblasts or cells of other lineages. Mazzocca et al. identified stem cells with 'tenogenic' potential from aspirates taken from the humeral head in a small study of 23 patients[23]. The stem cell characteristics were not extensively characterized and as the aspirated stem cells were not reinjected, the repair potential of these aspirated cells remains unknown.

Animal studies

Untreated Stem Cells

A number of studies have suggested that MSC treatment may improve both the volume and quality of regenerated tendons, with a greater propensity to heal via the generation of fibrocartilagenous tissue rather than scar tissue. MSCs treatment for lower limb tendons have resulted in improvements in a number of studies. Improved healing of the Achilles tendon enthesis has been noted following MSC treatment[24]. Achilles tendons injected with synovial derived MSCs demonstrated improved collagen fiber appearance as early as 1 week after treatment [25]. Bone marrow derived MSCs have been shown to improve the histological and mechanical properties of patellar tendons[26]. The histological improvement was limited to maturation of collagen fibers and cells, as no improvement was noted in the microstructure of MSC treated tendons. MSC augmented semitendinosus tendons were used to reconstruct ACLs and resulted in improved mechanical properties at 8 weeks, as measured by greater failure loads and stiffness[27]. Similarly, another study of MSC augmented tendons used to repair rabbit ACLs demonstrated improved histological and failure and stiffness properties at 8 weeks[28]. Some studies have suggested that MSC mediated healing may have a temporal effect, with greatest efficacy demonstrated during early phases of healing. Improved tendon mechanical and histomorphometric properties following MSC injection into rabbit Achilles tendons was only noted at 3 rather than 6 weeks, even though MSCs were still noted to be viable at 6 weeks[29].

MSC augmentation of rotator cuff repairs has also been investigated. The effects of MSCs derived from bone marrow, muscles and synovium on infraspinatus muscle regeneration were investigated in an ovine model of delayed rotator cuff repair[30]. MSC injection following six weeks of infraspinatus muscle retraction was found to significantly increase the contraction force, muscle vascularity and ratio of myocytes to adipocytes. Interestingly, the cambium layer of periosteal tissue contains high concentrations of MSCs and was sutured to rabbit infraspinatus defects, resulting in improved mechanical properties[31]. Amniotic derived MSCs have been seeded onto bioprosthetic scaffolds and used to successfully augment the repair of partial diaphragmatic tendon tears by improving the tensile strength and failure rates[32].

Treatment of racehorse tendinopathies with MSCs has received a great deal of interest[33]. Successful isolation of

MSCs from the sternum of racehorses has been demonstrated and expanded MSCs have been injected under ultrasound guidance into equine superficialis flexor digitorum tendons (SFDT), which is analogous to the human Achilles tendon[34]. In a small series, 9 out of 11 racehorses were able to return to racing and MSC treatment was associated with a reduced tendon reinjury rate and improved collagen fiber organization as measured by ultrasound[35]. A 2 year-follow up study of MSC therapy for overstrain injuries of the SFDT reported a reduction in reinjury rates in racehorses[36].

A concern with animal models of tendon damage is that the tendons are usually healthy, even if a defect is artificially created. Schnabel et al. attempted to assess the effects of MSCs on damaged tendons by using an equine model of collagenase induced tendinitis in SFDTs. While an improvement in tendon histological scores was noted in the MSC treated group, there was no difference in expression of ECM related proteins[37].

Questions have been raised as to whether improved tendon regeneration can be seen in the presence of any cells, rather than specifically in the presence of MSCs. Hankemeier et al., addressed this question by comparing the effects of human MSCs and fibroblasts on a patellar defect[38]. Histological improvements were seen in the MSC treated group at 10 days only, but not at 20 days and no effect was seen on the ultimate stress properties of tendons. Increased collagen 1 and 3 mRNA production was noted at 20 days in the MSC group but histological and mechanical properties were unaffected. The effect of MSCs on regeneration of mechanically damaged SDFTs were compared to embryonic stem cells (ESCs)[39]. ESCs showed greater survival and migration throughout the tendon compared to MSCs, suggesting that they may be a more effective source of stem cells. However, given ethical concerns surrounding ESCs, most research has shied away from this area in favour of MSCs.

While a number of animal studies have suggested that stem cells can enhance tendon healing, many studies did not measure tendon associated markers, making it difficult to characterize the true fate of any differentiated cells. No consensus has emerged from animal data about the ideal concentration or number of cells, or the most efficacious delivery modality. A number of *in-vitro* studies have attempted to address such questions.

Delivery of Stem Cells with Scaffolds

Finding an ideal carrier that will facilitate MSC localization and sustained function at sites of tendon damage may be essential to facilitate effective function. A rat model was used to study the effect of MSC delivery to rotator cuff defects using a fibrin carrier[40]. Whilst MSCs were shown to be viable in the rotator cuff, no improvement was detected in tendon composition, structure or mechanical strength. Fibrin glue has been used to coat allografts with MSCs that were used to repair rabbit ACLs, ultimately resulting in improved histological appearance and mechanical properties[41]. As well as fibrin, collagen gel and sutures have been studied as delivery vehicles for MSCs. Young et al. suspended MSCs into a collagen gel that was contracted onto pretensioned sutures and used these sutures to repair a rabbit Achilles tendon defect[42]. While the regenerated tissue varied from native tendon as it predominantly consisted of fibroblasts rather than tenocytes, it did result in improved mechanical load properties, tendon cross sectional area and collagen fiber alignment at 12 weeks. A similar study by Awad et al. delivered MSCs onto collagen matrices and contracted sutures, which resulted in improved mechanical properties and larger tissue volumes but no difference in histological appearance[43]. Poly (lactic-co-glycolic acid) (PLGA) coated with fibroblast growth factor has been shown to stimulate tenogenic differentiation of seeded MSCs[44]. MSCs seeded onto polyglycolic acid (PGA) sheets were used to repair rotator cuff defects in a rabbit model of infraspinatus tears ad produced superior histological, collagen 1 and mechanical properties compared to PGA sheets alone[45]. Yao et al. studied the effects of adding MSCs to sutures coated with intercellular cell adhesion molecule 1 and poly-L-lysine, in a rat Achilles tendon repair model. MSC enhanced sutures produced an early improvement in repair strength at 7 and 10 days, but this improvement did not last at later stages[46].

The scaffold material and surface properties may influence stem cell differentiation. MSCs were shown to preferentially differentiate into tendon stem cell precursors in the presence of collagen, whereas fibronectin scaffolds encouraged osteogenic differentiation[47]. A highly organized topographical surface reportedly enhances the expression of tendon-specific markers rather than osteogenic-specific markers[48]. Cryopreserved tendon allografts have been used as scaffold for culturing MSCs, and were successfully used to augment patella tendon defects in a rabbit model. Viable MSCs and improved histological properties were measured at 8 weeks. A number of synthetic materials have been trialed as delivery vehicles for stem cells, such as PGA, PGLA and PLA[49-51].

Direct Modulation of Stem Cells through Gene therapy

Mesenchymal progenitor cells can be manipulated to encourage a tenogenic fate. Plasmids encoding SMAD-8 and bone morphogenetic protein-2 (BMP-2) signaling proteins were injected into partially torn Achilles tendons in a rat model and resulted in improved tendon formation and healing[52]. Smad8/BMP-2 genetically engineered MSCs have also been used to repair Achilles tendon defects in mice. They produced a variable effect on mechanical properties by producing a significant increase in stiffness and elastic modulus, but no effect on ultimate load or maximum stress[53].

Identifying the appropriate key genes and the optimal delivery timing may be a key determinant of successful stem cell-mediated tendon healing. Attempts to recapitulate signals present during embryonic tendon formation have resulted in some promising results. Injection into the rotator cuff of MSCs transduced with scleraxis and membrane type 1- matrix metalloproteinase (MT1-MMP) improved the histomorphometric and mechanical properties of tendons after four weeks[54,55]. In contrast, injection of MSCs transduced with BMP-13 did not produce any improvement in regenerating tendons, which further supports the need to find provide appropriate cell signals in addition to stem cells[56].

Indirect modulation of Stem Cells via Growth Factors

In order to enhance the efficacy of stem cell use, there may be a role for modulation of stem cells with cytokines and cell signals, particularly as stem cell differentiation and ultimate fate is affected by culturing conditions and exposure to appropriate signals. Co-culturing bone marrow stromal cells with tenogenic substrates (postulated to be type-1 collagen of moderate rigidity, 30-50kPa) reportedly increased tenogenic differentiation[57]. The authors postulated that paracrine signaling between stromal cell populations was mediated by BMP-2 and the transcription factor Smad-8. MSCs can differentiate into tenocytes in the presence of the appropriate culturing conditions and exposure to cell signals and cytokines such as BMP-2, transforming growth factor $_3$ (TGF- $_3$), prostaglandin E_2 (PGE_2) and platelet rich plasma (PRP) releasate[58-61]. PRP specifically has been shown to increase the size and length of tenocytes, as well as increasing the proliferation rate and production of collagen types I and III[60]. However, selection and delivery of key growth factors at appropriate times may play a key role in determining their effectiveness in enhancing stem cell mediated effects on tendons. This was supported by results from a study by Martinello et al. which found that the combined use of blood derived MSCs and PRP did not have a synergistic effect on tendon healing in sheep, compared to MSC treatment alone[62]. The optimal combination of stem cells with growth factors and their appropriate doses still need to be determined for enhancing tendon healing.

Indirect Modulation of Stem Cells with Mechanical Loading

Mechanical loading of tendons at low strain rates has been proposed to encourage tendon healing[63]. Similarly, tendon derived stem cells have been shown to be respond to tensile loading by increasing BMP-2 expression as well as cellular alignment along the mechanical loading axis[64,65]. Furthermore, differentiation of tenocyte stem cells is reportedly influenced by the magnitude and direction of applied mechanical load[66]. A tenogenic fate was demonstrated for tendon stem cells following the application of low strain rates whereas higher strain rates encouraged stem cell differentiation down osteogenic, adipogenic and chondrogenic lineages[67].

These findings were replicated in a rat study of animals subjected to a treadmill running protocol[68] wherein Zhang et al. measured higher tendon stem cell proliferation and collagen production rates. The mechanical environment during culturing can also affect differentiation, as more rigid mechanical culturing substrates were found to favour osteogenic rather than tenogenic differentiation[57].

The relationship between mechanical loading and stem cells is likely to be a complicated one, as both intensity, magnitude and duration of loading are likely to influence differentiation. The length of refractory periods between loading of MSCs, as well as intensity and magnitude were shown to vary the balance between differentiation down either osteogenic or adipogenic fates[69]. Further research is required to try to delineate the effects of these variables and to determine an optimal loading schedule.

Clinical Application of Stem Cells To Tendon Healing

Despite wide spread interest in the application of stem cells to clinical settings, only a limited number of orthopaedic studies investigated the use of MSCs have been published to date. The most common application has focused on the effect of stem cells on encouraging bone regeneration. Stem cells have also been utilized during spinal surgery and for foot and ankle surgery with varying levels of success[70-72].

Few studies to date have studied the effects of MSCs on clinical tendon healing. MSCs were shown to improve patient related outcome scores and ultrasound tendon appearance following treatment of 12 subjects with refractory elbow epicondylitis[73]. 60 patients with patellar tendinopathy were treated with either skin derived tenocyte-like cells (N=33) or plasma (N=27)[74]. A significant improvement in clinical scores was reported by the group treated with stem cells, as measured by Victorian Institute of Sport Assessment (VISA) scores, in addition to a significant reduction in tendon thickness. Both treatment groups reported improvements in tendon hypoechogenicity and tear size.

The ideal source of stem cells for clinical applications remains undetermined. Local harvesting may avoid the need for donor site morbidity that is associated with iliac crest harvesting. Microfracturing local bone by drilling multiple burr holes may potentially release sufficient stem cells. This principle could be applied to the greater tuberosity during rotator cuff repairs. Similarly acromioplasties may release stem cells and may contribute to the fact that fewer rotator cuff tears are seen up to fifteen years following this procedure[75]. Alternatively, local tissue which is relatively easy to access may be used for harvesting stem cells, such as the synovium or adipose tissue[19].

No clinical studies to date have replicated the promising efficacy of stem cell mediated tendon healing reported in animal models. Many animal studies have cultured and expanded MSCs prior to reimplantation due to the low number of viable MSCs aspirated from bone marrows. Replicating this process for clinical use raises a number of significant concerns as the cells may undergo mutations and phenotypic drift, or potentially transmit zoonotic infections due to the culture medium utilized. MSC isolation techniques that do not require cell expansion are optimal. Such techniques are available in clinical practice already, wherein cells are aspirated and then concentrated within the operating theatre and then reimplanted within the same procedure[76].

A number of commercially available systems are now marketed as 'one-stop' cell isolation techniques. However, without adequate characterization of the implanted cells by measuring stem cell specific surface markers, or colony forming units, it is difficult to quantify whether these systems are producing effective numbers of MSCs. There is a need for an adequately powered randomized controlled trial to address whether stem cells are an effective treatment option for augmenting tendon healing.

Future questions to be addressed

The future of tendon repairs is already seeing an increasing interest in augmentation, whether it be through the use of biological therapies such as stem cells and growth factors, or with mechanical augmentation of repairs with patches.

A number of questions remain unanswered regarding the optimal source of stem cells, the concentration and delivery method. Different techniques for stem cell aspiration have been shown to affect the number and viability of harvested MSCs[77]. The correlation between increased concentrations of stem cells and tendon healing remain undetermined. Using higher concentrations has been shown to affect the effectiveness of bone healing[76,77]. Hernigou et al. have suggested that the total number of stem cells and their concentration plays an important role in achieving bone union, and similar principles are likely to apply to tendon healing. Ideal recommended stem cell numbers include a minimum concentration of 1000 cells per cm^3, although 100,000 cells per cm^3 is optimal in addition to a minimum total of at least 30,000 progenitor cells[77]. Awad et al. investigated whether impregnating sutures with different MSC densities would affect the repair of rabbit patellar tendon defects, and reported that no effect of cell density were noted on outcomes suggesting that higher cell concentrations do not always translate to superior healing[43]. Similarly, another study by Juncosa-Melvin et al. found no difference in seeding high or low concentrations of MSC densities onto repair constructs to augment patellar tendon defects in a rabbit model[78]. It will also be interesting to study the effects of current surgical and biological treatments on tendon stem cell populations. For example, in-vitro studies have suggested that commonly used dexamethasone impairs tenogenic differentiation of tendon stem cells[79].

The long term clinical safety of stem cell use remains unproven. Stem cells have the potential to exhibit tumour-like growth, differentiate into undesirable lineages, encourage ectopic tissue deposition or to modulate the immune system. One animal study reported that 28% of patellar tendons treated with MSCs resulted in ectopic bone formation, causing concern[43]. The addition of tendon derived stem cells with BMP-2 was shown to result in ectopic calcification[64]. One clinical study injected cultured autologous MSCs into peripheral joints (N=213) or intervertebral discs (N=13)[80]. No malignant transformations were noted at an average follow up time of 10.6 months, although 1 patient did develop cancer, which the authors stated was 'certainly unrelated'. Short term MRI follow-up in 45 patients at approximately 2 years did not reveal any tumour formation, although longer-term follow-up is required to confirm that MSC therapy is a safe procedure. Furthermore, during MSC culturing there is the potential for stem cells to undergo mutations or genetic drift, and the use of fetal bovine serum may result in transmission of zoonotic infections. Important clinical questions remain unanswered regarding clinical MSC use. Long term clinical follow up following MSC treatment is required to determine safety, and level I randomized control studies are required to address efficacy over other treatments.

Conclusion

Poor regeneration of tendons following damage and degeneration has encouraged the search for biological therapies to augment tendon healing. Stem cells therapies for enhancing tendon healing are an exciting new area of research. It is hoped that stem cells may help to recapitulate the appropriate signaling environment to produce regeneration of tendons rather than scar formation during healing. A number of regulatory, ethical and safety concerns have limited the use of stem cells to MSCs rather than more promising embryonic stem cells, although induced pluripotent stem cells are also being investigated. Achieving optimal stem cell efficacy may lie in direct and indirect modulation of stem cells through addition of cytokines and mechanical loading, or via genetic transfection with signals such as scleraxis or BMPs. Despite some promising preliminary animal and clinical studies, further research is required to determine whether stem cell therapies are actually an effective treatment option. Furthermore, a number of questions need to be addressed regarding the safety, optimal source, concentration and delivery vehicle for stem cells.

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