Stem cell therapy of tendinopathies: suggestions from veterinary medicine

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Summary

The ideal strategy for tendon healing has not been identified to date. Recently, the use of stem cells based therapy has been proposed, due to their ability to proliferate and to differentiate towards specific connective tissues lineages.
Embryonic stem cells should be considered the ideal cell source for regenerative therapies, but ethical factors limit their use in humans. Mesenchymal stem cells are more easily available and can be obtained by different sources. Amnion derived stem cells can differentiate towards all three germ layers, and can be used for allogeneic transplantation and stored thanks to cryopreservation.
In veterinary medicine, stem cells have been used with encouraging results for the treatment of the Superficial Digital Flexor tendinopathy in the horses. Considering that Superficial Digital Flexor tendinopathy is similar for pathogenesis and histopathology to Achilles tendinopathy in man, this experience can provide supportive data to encourage the use of regenerative therapy in humans.

Key words: stem cells, tendinopathy, Achilles tendon.

Introduction

Tendons are dense connective structures that transfer muscle force to bones; being frequently exposed to intense mechanical loads[1], they are prone to traumatic and degenerative injuries.

The interest for tendon disorders in scientific community has been prompted by three major considerations: the high prevalence, the poorly understood pathogenesis, and the deceiving results of different therapeutic approaches.
First of all, tendon injuries affect a large number of subjects both in humans and in horses[2]. The Achilles and patellar tendons are most commonly involved in humans[3], while Superficial Digital Flexor Tendon (SDFT) is most often interested in horses[4].
Secondly, despite a large amount of research, the pathogenesis of tendon disease is still debated. In the past, inflammation was considered the major determinant, and accordingly the term *Tendinitis* was used. Subsequently, it was observed that, in the late stages of the diseases, inflammation was absent, and degenerative features were exclusively found. For these reasons, the term *Tendinopathy* was suggested to emphasize that both inflammatory and degenerative aspects could be involved in the different pathogenetic stages of the disease[2]. The term *Tendinopathy* will be therefore used in the present paper.
Finally, it must be considered that, despite enormous efforts, the ideal strategy for tendon healing has not been identified to date. Several treatments have been proposed, such as the eccentric training in man[5], and controlled training schedules in horses[6]. Intra or iuxta lesional drug injection[7] or surgical management[8], depending on the site involved and the type of the lesion, have been also extensively used. This large range of solutions reflect the absence of a validated effective protocol enabling to induce a true regenerative process.
Recently a growing interest has been attracted by the use of stem cell based therapies. Peculiar features of these cells are the ability to proliferate and to differentiate toward different lineage pathways. The progression toward differentiation is maintained both by the intrinsic cell properties and the environmental niche where the stem cells reside. Different tendon progenitor cells have been investigated and used in preclinical and clinical settings, including mesenchimal stem cells and embryonic and placental stem cells[1].
However, several biological and clinical aspects concerning the ideal cell source, the number and the optimal timing of the engrafting procedure are not completely assessed to date.
The purpose of this paper is to review the relevant literature on this issue, focusing on experimental researches on animal models. Indeed, the experimental paradigm offers the possibility to thoroughly examine the explanted sample, whereas in humans very limited observations can be carried out by means of very small biopsy samples. Given the similarity of some animal tendinopathies with humans (e.g. SDFT lesions in the mid-metacarpal re-

gion and Achilles tendinopathy), these findings could result of great interest.

Mesenchimal Stem Cells

Multipotent cells in adult individuals originate from numerous mesenchymal tissues, including bone marrow, tendon, muscle and adipose tissue.

Bone marrow mesenchymal stem cells (BMSCs) were identified several years ago[9] and, to date, are probably the best known multipotent adult cells. They display adhesion molecules, specific cell surface markers, growth factors, and extracellular matrix molecules. Moreover BMSCs lack in MHC-II expression, disruption of T cell rejection mechanisms, and secrete anti-inflammatory mediators such as prostaglandins and IL-10[10]. Therefore they can be used, apart for autotransplantation, also for allogeneic procedures, which allow more efficient harvesting and expansion, but have the disadvantage of potential transmission of viral or prion vectors. It has been stated that BMSCs survive and maintain their characteristics after cryopreservation and thawing; therefore, they can be used "off the shelf" in emergency situations. However, once differentiated, the evidence about persisting immune-privileged properties is inconclusive. MHC-II antigens can still be detected intracellularly by western blotting, even though they are not expressed on the cell surface[11].
The exact mechanism exerted by BMSCs is not yet completely clarified, but there is mounting evidence that a paracrine effect follows the engrafting and differentiation of transplanted cells. In fact BMSCs secrete a variety of soluble autocrine and paracrine growth factors, which recruit more BMSCs, promote cell survival, and enhance the proliferation of endogenous connective tissue cells. The growth factors stimulate mitosis in tissue progenitors, induce angiogenesis, and reduce apoptosis[12].
However, BMSCs suffer some limitations, such as the low number of cells that can be obtained, the time needed for their expansion, the invasiveness of the surgical retrieval, and the skillness required for the sampling. These factors limit the diffusion of the technique.

Adipose derived stem cells (ASCs) for tendon repair have been recently considered. Adipose tissue could be one of the most suitable sources for cell therapy, because of its easy accessibility, minimal morbidity and abundance of stem cells. The large number of stem cells in this tissue could potentially eliminate the need for *in vitro* expansion[13]. However, the conventional method of isolation involves the enzymatic digestion and centrifugation, and therefore exposes the cells to mechanical trauma. For this reason, some author claims that the use of the explant culture, which is a simple, inexpensive and gentle method, could be preferred especially in cases of limited starting material[13]. Moreover, it has been shown that adipose tissue houses different subtypes of stem cells, which exhibit slight but significant differences in proliferative capacity and differentiation potential[14].
Indeed there are some difference in the secretion profile of different cytokines and growth factors, derived by various combinations of human ASCs populations in vitro, and it is unknown what could be the therapeutic potential of different combinations[15].

Tendon stem/progenitor cells (TSPCs) have been recently identified within tendon tissues. These cells exhibit universal stem cell characteristics, such as clonogenicity, high proliferative capacity and multi-differentiation potential[16]. Experimental studies in mice illustrate that, when injected into tendons, TSPCs stimulate tenogenesis more than BMSCs. Age has a marked influence on the number of TSPCs in tendon tissues, because, in aged animals, the proliferation rate is decreased, and cell cycle progression is delayed[17].

Embrionic Stem Cells

Embrionic stem cells (ESCs) can be isolated from the inner cell mass of the blastocyst, and are truly pluripotent cells in that they can differentiate into all the three germ layers (ectoderm, mesoderm and endoderm). Additionally ESCs can undergo genetic modification and differentiation into specific cell types and, by definition, can be incorporated into chimeric animals.
Saito et al.[18] have shown the potential of *in vitro* proliferation for more than 56 passages of equine ESCs lines. Due to these characteristics ESCs should be considered the ideal cell source for regenerative strategies, particularly for mesenchimal lineage since they can provide an unlimited supply of MSCs and connective tissue progenitors. Moreover, a direct differentiation of ESCs into tendons in humans and mice has been recently demonstrated[19].
However, the destruction of human embryos required for their harvesting and the high rate of tumor formation following transplantation limits their use in human tendinopathies[18, 19].

Amnion derived stem cells

MSCs have been derived from Umbilical Cord Blood and amnion (Amniotic Mesenchimal Stromal cells: AMSc)[20]. MSCs isolated from amniotic fluid, umbilical cord blood and Wharton's jelly in the horse, show similar biological characteristics: all cell lines expand rapidly in culture, exhibit multi-differentiation potential, are positive for CD90, CD44, CD105, and negative for CD34, CD14 and CD45[21]. Moreover, from this peculiar source, Amniotic Epithelial Cells (AECs) can been obtained, and these ones are to date considered a promising source of stem cells to be used in tendon tissue engineering[1].
The amnion is derived from the epiblast as early as 8 days after fertilization, two weeks before gastrulation. Since gastrulation is considered the time point for cell commitment, it has been suggested that amniotic cells may retain the pluripotent properties of early epiblast cells[22]. Therefore, they can differentiate into cell types derived from all the three germ layers[22] and can display direct differentiation towards cells of the osteogenic, chondrogenic and adipogenic lineages[23].

To date, AECs have been studied extensively[20]. They show a typical epithelial appearance and can be maintained for 2-6 passages before ceasing proliferation[24]; their pluripotency is illustrated by the expression of molecular markers of pluripotent stem cells, including octamer-binding protein-4 (OCT-4), SRY-related HGM-box gene 2 (SOX-2) and Nanog[22, 23].

In a study on AECs isolated from horse amniotic membrane, Lange-Consiglio et al.[25] found that these cells were positive for the expression of specific embryonic markers (TRA-1-60, SSEA-3, SSEA-4 and Oct-4), and mesenchymal stem/stromal cell markers (CD29, CD105, CD44 and CD166).

Our group has recently demonstrated that ovine AECs are similar to ovine MSCs and human AECs[26], due to the expression of adhesion molecules (CD 29, CD 49, CD 58 and CD 166), and the wide expression of stemness markers (Oct ¾, Sox 2 Nanog and TERT)[26, 27]. Other very interesting characteristics displayed by amniotic cells, shown both in human and ovine species, are the low immunogenicity and the ability to induce immuno-tollerance[1]. Low immunogenicity has been demonstrated also by xenografts studies, transplanting human AECs into neonatal swine and rats, in the eye and the lung[28]. The reasons of the low immunogenetic potential have been partly clarified. Hori et al.[29] reported that AECs do not express HLA-A, B, C, and DR antigens, but express HLA-G on their surfaces. HLA-G molecule displays, at least, four inhibitory functions relevant to immune responses: 1) it can bind directly to inhibitory receptors found in NK cells and other leucocytes; 2) it expresses the appropriate leader peptide for binding to HLA-E, which will in turn inhibit the NK cells via their CD94/NKg2 receptor; 3) it can induce apoptosis of activated CD8+ Tcell; 4) it can inhibit CD4 + T cell proliferation[30].

This feature is extremely relevant, suggesting the opportunity of the use of these cells for allotransplantation. The creation of a xenogeneic chimera *in vitro* by Tamagawa et al.[31] further supports this assumption.

Experiments from our laboratory clearly show that ovine AECs can differentiate into bone tissue and tendon tissue, both *in vitro* and when implanted into live animals[1, 26, 32]. In particular, using an experimental model of tendinopathy, we observed that, when grafted into the tendon defect, AECs can survive for at least 28 days, with a percentage of phagocytosed labeled cells never exceeding 35%[1]. Moreover, implanted cells were able to synthetize ovine Type I collagen, thus suggesting the ability to differentiate into tenocytes also after *in vivo* transplantation[1].

Since ovine model is considered a very useful model for translational studies in humans, these findings are of great speculative interest.

Finally, another positive aspect is the absence of tumorigenicity, as shown after the injection of 1 million cells per site into the rear muscles and/or the testis of severe combined immunodeficiency mices[22].

Use of Stem Cells in Veterinary Medicine

Preliminary studies in different laboratory animals (rats and rabbits) tendons (patellar, rotator cuff), and experimental models of tendinopathy (collagenase, surgical defects) have shown some improvement in structure and strength, after implantation of stem cells isolated from different sources[33-35].

These promising results have stimulated their use in veterinary medicine.

Spontaneous tendinopathies, mainly of the SDFT, are very frequently observed in racehorses, due to hyperextension of the metacarpophalangeal joint during weight-bearing[36]. Epidemiological data suggest that approximately 25% of racehorses are affected by the disease[37] and that the prevalence increases with age[36]. This suggests that, while injury appears to be spontaneous, occurring most commonly during high speed exercise, it is preceded by degenerative changes within the extracellular matrix[38]. Usually, the tendon repairs via a process of fibrosis[39], and becomes stiffer, with important consequences for the animal in terms of reduced performance, and risk of reinjury. Therefore, the economic impact may be relevant. During the repair process, the cells involved in the synthesis of new tissue are believed to be tissue-specific progenitor cells, that are most likely to reside in the endotenon tissue, between the collagen fascicles and adjacent to the vasculature[39]. These cells have been demonstrated abundantly in young tendons[40], but mature equine tendons do not appear to possess a substantial subpopulation of tissue-specific progenitor cells. Therefore, the implantation of autologous MSCs could have the potential of regenerating or improving the repair of the tendon.

Studies in horses have been performed using ASCs recovered from the tail-head[41], or from bone marrow[42], and have shown promising results in collagenase-induced experimental tendinopathy.

However, disappointing results have been obtained by Caniglia et al.[43], after the implantation of autologous MSCs derived from bone marrow into a surgically created central core defect in the SDFT. In this experimental model no significant differences were observed between treated and control limbs for collagen fibril mass and size.

Few data have been published on the treatment of naturally-occurring tendinopathy[44-46].

In the largest cohort[45], 141 racehorses, with spontaneous occurring SDF tendinopathy, were treated with autologous BMSCs and followed-up for a minimum of 2 years after return to full work. The reinjury percentage of all racehorses undergoing MSCs treatment was 27.4%. Despite the historical comparison with previous published studies does not represent the best of clinical evidence, this percentage resulted significantly less than that observed in other series where stem cells[44] or other treatments[47, 48] were used.

Safety was assessed clinically, ultrasonographically, scintigraphically and histologically in a cohort of treated cases, and no adverse effects of the treatment were observed, with no aberrant tissue on histological examination.

New approaches for the treatment of spontaneous tendinopathies come from the use of AECs. In the Lange-Consiglio[25] experiments, AECs isolated from the horse amniotic membrane, were allogeneically injected into

three horses with tendon injuries, resulting in a quick reduction in tendon size and cross-sectional area.
Preliminary research by our group[49] has shown that ovine AECs, transplanted from sheep to horses with SDF tendinopathy, survive in pathologic tendons. This conclusion comes from the observation that cells marked with a vital probe are present in the graft more than 40 days after transplantation, and is a clear demonstration of their very low immunogenetic potential. In addition, these cells seem to have a positive role in the healing process. Indeed, forty days after implantation, deposition of collagen, organized into parallel arrays of fibers, was observed, and sonography showed a more homogeneous echotexture of the treated areas without neovessels.
The exact role played by the implanted cells in tendon healing is still incompletely known. They may either differentiate into tenocytes and synthesise the tendon matrix themselves, or they may act in a paracrine or trophic fashion to provoke resident cell populations to synthesise new tissue. In addition, MSCs are thought to have anti-inflammatory effects via their inhibition of T cell mediated responses[10].

Conclusions

To date, the studies in humans using stem cells in the treatment of tendinopathies are very few and do not allow any firm conclusion.
In the systematic review, performed recently by Ahmad et al.[50], 5 clinical studies met the inclusion criteria: only 1 was a randomized controlled trial, which showed that skin-derived tendon cells had a greater clinical benefit than autologous plasma. One cohort study showed the benefit of stem cells in rotator cuff tears and another in lateral epicondylitis. Two of the human studies showed how stem cells were successfully extracted from the humerus and, when tagged with insulin, became tendon cells.
Given this limited experience, data gained from treating spontaneously-occurring tendon injuries in horses can provide supportive data to encourage the translation of regenerative technology into the human field. Indeed, very interestingly, the lesions of SDFT in the mid-metacarpal region have many similarities with Achilles tendinopathy in man[4].
However, it is wise to avoid premature enthusiasm. A lot of questions remain still opened. First of all, it is necessary to establish what stem cells are provided of the best repair potential, for auto or allogeneic transplantation. Therefore, there is urgent need of identifiyng a plentiful, safe, economic and ethically acceptable stem cell source[20].
At this regard, human term placenta has turned great attention as a possible non controversial source of progenitor/stem cells. The robust literature on amnion derived stem cells depicts the unique features of these cells: their ability of differentiation towards all three germ layers, and the immunomodulatory effects suggest the hypothesis of their use in allogeneic transplantation settings. Moreover, the possibility to store amniotic membrane *in toto*, as well as amniotic cells, thanks to cryopreservation, allows to undertake the treatment in the early phases of the healing process, which is considered to induce the best functional improvement[1]. For these reasons, Evangelista et al.[20] stated that human term placenta represent a prime candidate, as it is available in nearly unlimited supply, is ethically problem-free and easily procured.
Besides that, important questions to be answered are the number of cells to be injected, and the characteristics of lesions which can have better advantage by treatment. The use of adjuncts can potentially enhance stem cell therapy: among them, the addition of platelet rich plasma, which can provide a scaffold capable of promoting the survival of implanted cells by protection, nutritional support, and growth factors supply, and the mechanical stimulation, by means of structured exercise protocols.

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