Cell-based Therapies for Tendon and Ligament Injuries

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Overstrain and traumatic tendon and ligament injuries are common in the horse and, for the most part, heal (repair) naturally by the formation of scar tissue. However, the scar tissue formed in this repair is functionally deficient compared with normal tendon, which has important consequences for the animal in terms of reduced performance and a substantial risk of reinjury, despite the multitude of treatments that have been proposed. Regenerative medicine offers the prospect of restoring normal, or close to normal, structure and function to an injured organ, thereby resulting in a successful restoration of activity without the risk of reinjury. Regenerative medicine aims to harness the combined effects of a cell source, scaffold support, and anabolic stimulus to facilitate the healing of the injury.\textsuperscript{1}

There are multiple choices for the selection of a cell source for regenerative medicine, and at this time it is not clear which source will prove to be therapeutically optimal. A logical source of cells for making new tendon tissue would be the tenocytes.

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themselves. However, the biopsy of tendon to prepare and propagate cells leads to formation of a secondary lesion at the donor site, an unacceptable option for flexor tendons in the horse. In addition, cells derived from different tendons do show different characteristics in culture, which may compromise their effectiveness when implanted into other tendons. Alternative sources of differentiated fibroblasts under investigation include dermal and ligament fibroblasts. Ligament fibroblasts not only have potential advantages in that they are often more metabolically active than other fibroblasts but also have different cell characteristics that may not be appropriate for use in tendon repair. However, the cells carrying the greatest hope for effective therapy are stem cells. Stem cells are defined as cells capable of differentiating into different cell lines and the 2 most studied are embryonic stem cells (ESCs; those derived from the embryo and capable of differentiating into every cell type in the body and therefore truly pluripotent) or mesenchymal stem cells (MSCs; those derived from postnatal tissues with multipotential capabilities to derive cells of mesenchymal origin). The terminology for these cells is not standardized and the terms mesenchymal stem, stromal, and progenitor cells are used interchangeably.

MSCs are generally found in varying but sparse numbers in many postnatal tissues and frequently reside as perivascular cells, whereby they likely play a role in normal turnover and tissue maintenance. MSCs have been isolated from a wide variety of tissues, of which the most established sources are bone marrow and fat. Other sources potentially useful for tendon and ligament cell therapies include umbilical cord blood cells, which potentially span the divide between fetal and postnatal stem cells and therefore could have greater regenerative potential. Furthermore, they can be easily recovered at birth and stored for future use. However, although umbilical cord blood is a relatively rich source of hematopoietic stem cells, studies have not consistently shown that cord blood is a reliable source of MSCs, and considerably more work is needed in this area before these cells can be advocated for clinical use. This article focuses on the use of stem cells derived from embryos, bone marrow, fat, and tendon and their potential for the treatment of tendon and ligament injuries.

FEATURES OF TENDON AND LIGAMENT INJURY THAT LEND ITSELF TO CELL THERAPY

Equine digital flexor tendon strain injuries provide many of the additional elements required for tendon tissue engineering. The lesion manifests within the central core of the tissue, thus providing a natural enclosure for implantation that, by the time of stem cell implantation, is filled with granulation tissue, which acts as a scaffold (Fig. 1). Tendon lesions have the added advantage of being highly vascularized and therefore capable of nutritional support to the implanted progenitor cells. The anabolic stimulus is provided by the cytokine and mechanical environment, which are potentially important stimuli for differentiation, in the intratendinous location of the cells and augmented by the suspension of MSCs in growth factor–rich solutions, such as bone marrow supernatant, which we have shown to have significant anabolic effects in vitro.

Postinjury, tendon does not exhibit restrictions of cellular infiltration but those cells actually involved in the synthesis of new scar tissue are mostly locally derived cells. Most tissues have a small population of precursor cells (tissue-specific progenitor cells) that function to replenish cells lost through natural turnover and aid in repair after injury. Evidence of multipotency has been shown for cells derived from young tendon; however, in adult tendon, investigators have been unable to demonstrate the presence of a cell subpopulation capable of differentiating into multiple cell
lineages (osteocytes, adipocytes, chondrocytes), as with bone marrow–derived cells. This limitation may explain why the influence of adult tendon progenitors in the repair process is restricted and results in natural repair inferior to normal tendon.

**POTENTIAL THERAPEUTIC MECHANISMS OF STEM CELLS**

The goal of using stem cells is to engineer new tendon tissue using cellular synthetic machinery. This goal can be achieved either via a direct contribution through differentiation into tissue-specific cell phenotypes and the production of tissue-appropriate extracellular matrix products or indirectly by trophic effects through the production of bioactive proteins, such as growth factors, antiapoptotic factors, and chemotactic agents.\(^8\)\(^-\)\(^10\) In addition, recent studies have suggested an antiinflammatory role of implanted stem cells. Animal model studies have demonstrated that MSCs are hypo-immunogenic and inhibit the activation of T and B lymphocytes and natural killer cells.\(^11\)\(^,\)\(^12\) The precise mechanism of the antiinflammatory effect of these cells is largely unknown, although a combination of this activity along with an antiapoptotic effect, additional recruitment of local multipotent stem cells, stimulation of vascular ingrowth, and the liberation of growth factors could all contribute to tissue repair.\(^4\)\(^,\)\(^13\)\(^,\)\(^14\)

It is not known which of these actions occur after stem cell implantation, although current opinion favors the paracrine actions as being most important.\(^15\)
**Bone Marrow–Derived MSCs**

Bone marrow contains a hematopoietic stem cell population, from which the cellular components of blood are derived, and a stromal network of fibroblastlike cells. Among these stromal cells, there is a subpopulation of multipotent cells, the MSCs,\textsuperscript{16} that are able to generate mesenchyme, the mass of tissue that develops mainly from the mesoderm of the embryo. These MSCs represent a small fraction of the total population of nucleated cells in bone marrow from human beings and cats, and only 0.001% to 0.01% of mononuclear cells isolated from a Ficoll density gradient of bone marrow aspirate are MSCs; this is presumed to be similar in horses.\textsuperscript{17}

Bone marrow–derived MSCs have been shown to be multipotent. In addition to self-replication and adherence to plastic in culture, they are able to exhibit osteogenic, adipogenic, and chondrogenic differentiation with appropriate growth factor stimulus. Thus, the presence of MSCs is frequently demonstrated by the ability of a derived cell population to differentiate into osteoblasts, chondrocytes, and adipocytes, capable of producing bone, cartilage, and fat, respectively, in vitro. In addition to characterizing these cells by their ability to differentiate into distinct cell lineages, MSCs can be further characterized by cell surface markers.\textsuperscript{18} However, their use in horses has been hampered because many of the positive stem cell markers described for other species use antibodies that show little or no cross-reactivity. The ability to select tendon tissue–promoting MSC populations by cell surface markers, with or without further modification, may be important for optimizing this therapy in the future.

We have chosen to harness the action of MSCs recovered from bone marrow because of ease of recovery and minimal donor site morbidity and, because these stem cells can be recovered from adult tissue, this source allows autologous reimplantation, which carries fewer regulatory and safety issues. Furthermore, in comparative experiments assessing multipotency, bone marrow–derived MSCs frequently outperform MSCs from other sources.\textsuperscript{19–21} We have therefore hypothesized that the implantation of autologous MSCs, in far greater numbers than are present normally within tendon tissue, will improve the repair of the tendon both structurally, as shown by optimizing mechanical properties, organization, and composition, and functionally, as measured by reduced reinjury rates.

**Technique for the recovery of bone marrow–derived stem cells**

Bone marrow is recovered in heparinized containers from the sternum (or, less commonly, the tuber coxae) under standing sedation. Marrow aspiration is generally performed within 3 months of injury, when there is an enclosed area of disruption still visible ultrasonographically. The aspirate is transferred to a laboratory in specially designed containers for culture and expansion of MSCs, which yields an enriched rather than a pure cell preparation. After approximately 3 weeks, the cultured cells are shipped back to the veterinarian (usually $10^7$ to $10^8$ cells, although this dose is increased empirically up to $50 \times 10^6$ if lesions are very large) and implanted into the damaged tendon of the same horse under ultrasonographic guidance (Fig. 2). The cells are suspended in citrated bone marrow supernatant for implantation so that no foreign material (such as fetal bovine serum) is implanted and to gain potential beneficial effects of the rich mixture of growth factors present in the supernatant.\textsuperscript{6}

**Delivery, engraftment, and survival**

We have shown in our laboratories that equine MSCs, cultured on fresh acellular equine tendon sections, not only survived, proliferated, and migrated into the matrix but also expressed extracellular matrix genes, although their expression was less than that of tenocytes cultured on the same tendon matrices.\textsuperscript{22,23} Longer culture times
In vitro, we and others have been able to demonstrate that the implanted cells survive within the tendon after implantation in relatively low numbers for up to 4 months (Fig. 3). However, although this technique allows high numbers to be delivered directly to the site of injury, it limits the use of the cells to distinct constrained lesions. To date, we are not able to treat contralateral tendons (which are likely to have a pathological condition but may not have a core lesion) or surface defects (e.g., intrasynovial tendon tears). Intrasyynovial administration may be an option for surface defects, and regional administration may also be possible, whereas systemic administration needs to address issues of homing to the injury site and engraftment.

**Safety**

The implantation of a cell type that is capable of self-renewal and had been implicated in the pathogenesis of certain cancers suggests a possible risk of neoplastic transformation. However, histopathologic examination of 17 tendons or ligaments from post-mortem samples obtained from 12 horses that had undergone MSC implantation did not reveal any abnormal or neoplastic tissue. Instead, there was evidence of organized

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**Fig. 2.** Transverse (A) and longitudinal (B) ultrasonographs immediately after cell implantation showing the location of the injected cell suspension by the presence of air bubbles (arrows).
crimped collagen fibers with minimal presence of inflammatory cells. We have not conducted extensive postmortem examinations on tissues at remote sites (such as the lungs), but there have been no reports of clinically significant abnormalities of other body systems after implantation.

Reported adverse reactions after the treatment of more than 1500 clinical cases have been rare. Only 1 horse developed peritendinous mineralization 2 years after MSC implantation; this may have been related to the original trauma rather than a consequence of the cell implantation. Needle tracts are common in the first few months after implantation (Fig. 4), although it is unlikely that they adversely affect the outcome, given their focal nature; they usually resolve within 3 months. Gamma scintigraphy was performed 3 months after implantation in 6 horses but this did not demonstrate any bone formation within the treated tendons, suggesting that this is an unlikely consequence of MSC implantation.

Four further cases have experienced acute inflammatory reactions: one was mild and transient and thought to be a bandage reaction, whereas the other 3 cases were associated with intrasynovial administration, which is an off-label use for the preparation. It is possible that these 3 cases were the result of infection through contaminated bone marrow supernatant. An extra filtration step is now included in the preparation to minimize this risk.
**Efficacy**

In vitro, MSCs cultured in 2- and 3-dimensional matrices can be induced to synthesize matrices with some of (but not all) the characteristics of tendon extracellular matrix. Equine MSCs can synthesize an abundant and remarkably well-structured matrix when cultured in vitro in a bioreactor within the coagulated supernatant of bone marrow. However, although several reliable determinants of osteogenic, adipogenic, and chondrogenic differentiation are available, demonstration of tenogenic differentiation is more problematic, partly because an effective tenogenic stimulus has not been well defined and fewer definitive tenocyte phenotypic markers have been identified. Tenocytes in culture have a fibroblastic morphology that is similar to MSCs and so cannot be identified from appearance alone. Collagen type I is the primary protein synthesized by tenocytes, but this does not distinguish these cells from fibroblasts capable of producing connective tissues, including scar tissue, in which deposition of collagen type III occurs in significant quantities. The synthesis of the glycoprotein cartilage oligomeric matrix protein (COMP) provides a more discriminating analysis but it too is not specific to tendon, although COMP does have a restricted distribution in tissues primarily designed to withstand load (eg, cartilage, tendon, and fibrocartilage). The use of a signature profile of synthesized extracellular matrix proteins enables a better discrimination between most musculoskeletal tissues. In addition, the transcription factor scleraxis and the transmembrane protein tenomodulin are selectively expressed by partially differentiated mesenchymal precursors of connective tissue and tendon; these might therefore be indicative markers of the tenocyte lineage. New markers of tendon differentiation will add to our ability to identify tenocyte lineages.

In vivo, MSCs have been implanted into surgically created tendon defects in multiple in vivo experiments using laboratory animal models, with almost universally positive outcomes. Most of these studies used surgically created defects in rabbit or rat tendons and have showed variable regeneration of new tendonlike tissue in defects implanted with MSCs in a biodegradable scaffold (collagen gel, Vicryl (polyglactin 910; Ethicon, Summerville, NJ) knitted mesh, or fibrin glue) as assessed by histology or simple biochemical assays. However, not all have shown an improvement in microstructure, and, because allogenic cells were implanted, an inflammatory reaction persisted. Furthermore, the implanted cells exhibited a fibroblastic morphology but were not fully characterized as tenocytes. In more recent studies, MSC implantation was associated with both improved strength and quality of reparative tissue (determined by collagen type I/collagen type III ratio). Thus, MSC-seeded constructs implanted in vivo are able to integrate into the tissue and synthesize tissue-specific extracellular matrix; however, it is unclear which factors are initiating this functional differentiation.

Although it is possible to demonstrate that implanted cells do survive, it has not yet been possible to demonstrate that the implanted cells actually synthesize, or induce the synthesis of, a tendonlike matrix or identify the mechanisms of these effects. Furthermore, it is not clear whether such a mechanism may arise from a truly regenerative response or whether any benefit arises more from an influence on the inflammatory process that follows injury. Recently, experimental studies in horses using a collagenase-induced injury have demonstrated significant improvements in some, but not all, parameters. A surgical model is as an alternative to test cell therapies, although initial data have not shown a significant effect on collagen fibril diameter (C.J. Caniglia, M.C. Schramme and R.K.W. Smith, unpublished data, 2011). However, in a controlled study of MSC versus saline treatments in naturally occurring tendinopathy in horses, mechanical testing and biochemical and molecular analyses of the new tissue synthesized after treatment demonstrated that stem cell treatment seemed to
improve histologic scores of the healing tissue toward that of normal tendon compared with saline injection (Young and colleagues, unpublished data, 2011).

Ultrasonographic appraisal of treated cases show a rapid filling in of hypoechoic lesions, although the longitudinal striated pattern of the regenerated tendon rarely returns completely to normal (Fig. 5). In the analysis of 113 racehorses treated with bone marrow–derived MSCs, the reinjury percentage was 27.4%, with the rate for flat (n = 8) and National Hunt (n = 105) racehorses being 50% and 25.7%, respectively. This recurrence rate was significantly less than those published for National Hunt racehorses treated in other ways ($P<.05$ vs Ref.37; $P<.01$ vs Ref.38). No relationship between outcome and age, discipline, number of MSCs injected, and injury to implantation interval was identified. In further support of this improved outcome, reinjury rates in sports horses showed a similar reduction after MSC administration.

A more limited number of cases with injuries to other tendons and ligaments have been treated with bone marrow–derived MSCs. For lesions present within a tendon sheath, cell implantation is usually done after tenoscopic evaluation to ensure that there are no surface defects through which the cells could leak.

**ESCs**

ESCs carry the major hopes of stem cell science because of their seemingly unlimited capability to generate all functional adult cell types. Permanent ESC cell lines can be established and maintained in a pluripotent undifferentiated state. At the blastocyst stage of development, the preimplantation mammalian embryo separates into the inner cell mass (ICM) cells of the putative embryo and the trophectoderm cells that will eventually form the outermost layer of the fetal placenta. Appropriate

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**Fig. 5.** Ultrasonographic appearance of a core lesion in a superficial digital flexor tendon treated with bone marrow–derived MSCs. There has been rapid filling in of the lesion, but the longitudinal pattern has not completely returned to normal.
environmental stimuli can cause ICM cells to differentiate into the cell types of all 3 germ layers: ectoderm, endoderm, and mesoderm, both in vitro and in vivo. Furthermore, it has become apparent that the culture requirements for proliferation and maintenance of ESCs in an undifferentiated state and the nature of the stimuli required to induce ESCs to transform into specialized end-stage cells of specific phenotype vary between humans and various animal species. In addition, as genetic manipulations can be performed and confirmed in culture, ESCs have become a powerful tool for the generation of reporter cell lines or gain-of-function/loss-of-function disease models.

Because the generation of ESC cell lines necessitates the destruction of an embryo, the development of these cells for clinical use has been associated with considerable ethical issues, especially in the human context. These issues may be, but are not necessarily, less ethically problematic for equine use. In an attempt to circumvent these issues, the exciting recent discovery of induced pluripotent stem (iPS) cells, derived from transfection of somatic (adult) cells with specific transcription factor genes, represents a possible alternative. iPS cells have been derived from equine cells, but considerably more research is needed before these cells will be regulated sufficiently well to be used effectively and safely in clinical cases.

**Use in horses**

A recent study has reported the use of putative ESCs in the treatment of damaged equine flexor tendon. In this study, the survival and distribution of ESCs were compared with those of MSCs. ESC persistence was higher than that of MSCs in the damaged superficial digital flexor tendon (SDFT), with numbers remaining at a constant level over 90 days. This could occur because the ESC population was either proliferating or was maintained by a balanced rate of proliferation and death, but it does suggest that ESCs respond positively to the tendon environment. Furthermore, the high survival rate of the ESCs coupled with minimal leukocyte infiltration suggests that the ESCs were not recognized as foreign by the host immune system. The different effects of ESCs and MSCs on tendon regeneration are not clear in this study, and future studies need to address which of these cell types will be of the most therapeutic benefit.

Although there are concerns that ESCs cannot be used for direct transplantation because of the risk of teratoma formation, no equine studies have reported this consequence. The failure of equine ESC-like cells to generate teratomas in an immunoprivileged site could be an experimental failure or a peculiarity of equine ESCs. This finding remains difficult to explain, given that ESC implantation in other species, under similar conditions, produced teratomas. It should also be stressed that teratoma formation at sites other than the implantation site in a very large animal and in a relatively short time frame is difficult to assess. Further investigation on the consequences of equine ESC transplantation is required to determine whether this anomaly is because of a peculiarity of equine pluripotent cells or because the ESC-like cells tested were not true ESCs.

ESCs offer tremendous potential for regenerative therapy in tissues with an inherently limited capacity for self-repair. However, it is clear that more controlled studies are needed to show efficacy with respect to optimizing tendon and ligament repair to enable clinical use of ESCs with success.

**Fat-Derived MSCs**

Adipose tissue provides an alternative source of cells capable of multipotential differentiation. These cells have gained importance because adipose tissue is readily
accessible in large quantities and adipose tissue–derived MSCs have been shown to be largely indistinguishable from bone marrow–derived MSCs. However, some studies have shown these cells to differentiate less capably into specific cell lineages.\textsuperscript{19–21}

\textbf{Technique for the recovery of fat-derived MSCs}

Adipose tissues can be harvested from several sites in horses, including inguinal, sternal, and gluteal regions. The area over the dorsal gluteal muscles, at the base of the tail, is the most accessible in standing horses, and it can provide significant quantities of tissue, although there can be relatively little fat available in some highly athletically tuned thoroughbred racehorses. The horse is sedated, the area over the dorsal gluteal muscles is aseptically prepared, and skin and subcutaneous tissues are desensitized by local infiltration of 2% lidocaine using an inverted L-block. An incision of approximately 10 to 15 cm in length is made parallel to and approximately 15 cm abaxial to the vertebral column, exposing a layer of adipose tissue between the skin and musculature. Approximately 5 to 15 mL of adipose tissue can be harvested over the superficial gluteal fascia, and the skin incision is then sutured with nylon material.\textsuperscript{14,21}

To isolate the cells, the adipose tissue is finely minced and digested with a collage-nase solution prepared in a suitable tissue cultured medium. Tissue digestion can be performed over 3 to 6 hours or overnight (18 hours), depending on need and convenience. After digestion, the nucleated cell fraction is separated and concentrated. These adipose-derived nucleated cells (ADNCs) have been used immediately for injection into tendon lesions\textsuperscript{13} or have been subjected to further expansion in cell culture, which may also enrich the stem cell fraction, similar to the technique used for bone marrow cells, before implantation as adipose-derived MSCs (AD-MSCs).\textsuperscript{14}

It is important to recognize the difference between these 2 approaches. The nucleated cell fraction released from the adipose tissue, referred to as the stromal vascular fraction (SVF), is a mixed population of cells that includes endothelial cells and preadipocytes and a relatively low number of MSCs.\textsuperscript{21} However, because SVFs have not been expanded in culture, they are termed minimally manipulated and are therefore subject to less regulatory issues in human medicine. In contrast, AD-MSCs expanded in the laboratory, although involving greater cost and labor, have the potential advantage that a greater number and a more homogeneous MSC population are generated. To date, there have been no reports of adverse consequences after ADNC administration, as opposed to a more purified stem cell preparation, although the relative therapeutic efficacies of these cell preparations have not been established. It is not apparent from the literature which of these preparations is preferable for tendon healing,\textsuperscript{13} and there are no published studies evaluating the optimal number of AD-MSCs that should be used in the treatment of tendinitis. It is possible that optimization of the AD-MSC dose used in tendon therapy could improve the future results of such studies.\textsuperscript{32}

\textbf{Efficacy}

There are only a few controlled studies investigating the use of AD-MSCs for the treatment of tendon injuries in horses. Analysis of the ultrasonographic appearance of the tendons revealed no significant differences between the ultrasonographic parameters of limbs that received AD-MSCs or ADNCs and their controls. Scores for linear collagen fiber pattern improved in both groups in these studies.\textsuperscript{13,14} However, histologic scoring showed that the lesions treated by AD-MSCs were more organized and had a more uniform tissue repair compared with the control limbs, including lower cellularity in the tendon, less inflammatory infiltrate, lower fibroblastic density, greater
parallel arrangement of the fibers, larger extracellular matrix deposits, and greater type I collagen expression.\textsuperscript{13,14,16} However, no significant differences were evident in the immunohistochemical assessments of cell proliferation and type I collagen spatial organization, although there was a reduction in the formation of type III collagen in the tendons that received treatment.\textsuperscript{13}

Expression of COMP was significantly increased in ADNC-injected tendons. Concentration of COMP in equine digital flexor tendons has been positively correlated with ultimate tensile strength and stiffness in young adult normal tendons, which suggests that COMP concentrations may be linked to organization of the tendon matrix.\textsuperscript{51} This increase in expression of COMP messenger RNA (mRNA) in the ADNC-treated tendon could be related to the improvements in tendon architecture and, consequently, in tendon regeneration.\textsuperscript{13}

\textbf{Bone Marrow Aspirate}

The first attempts to harness MSCs from bone marrow for the treatment of tendons and ligaments utilized the direct injection of neat bone marrow from the sternum.\textsuperscript{52} This strategy was reported to be successful primarily for the treatment of suspensory ligament disease, although the outcomes were derived from an uncontrolled clinical case series. In this technique, bone marrow aspirate is collected from the sternum, followed by direct injection of relatively large volumes (usually 10–40 mL) of the heterogeneous mixed-cell population into the tendon/ligament lesion. If the aspiration is performed efficiently, anticoagulant is not necessary. This further simplifies the direct aspiration–injection approach. The advantages of this procedure are the simplicity of the technique, the ability to perform the procedure immediately at the time of diagnosis, and relatively low cost, but the disadvantage is the injection of a heterogeneous mixed-cell population, with few stem cells. Furthermore, there is some concern about the use of the bone marrow aspirate in tendons because of the potential for mineralization (\textbf{Fig. 6}), although this has not been reported to be a common finding by users of this technique. This technique has largely been superseded by more specific techniques designed to supply a more pure population and higher number of MSCs. Using a method analogous to the adipose tissue stem cell technique, the nucleated cell population from bone marrow (bone marrow mononuclear cell fraction [BMMNC]) or bone marrow aspirate concentrate can be recovered by centrifugation. These preparations have a reduced proportion of MSCs (and contain mainly white blood cells) but allow horse-side preparation that optimizes timing and cost of treatment.

The clinical effect of these cruder techniques might be due to bioactive substances in the noncellular fraction, such as growth factors produced by cells or platelets,\textsuperscript{17} or the potential of the small number of MSCs or other cells.\textsuperscript{21} The effect of BMMNC administration has been assessed in a collagenase-induced experimental model of tendon healing in horses\textsuperscript{34} in which BMMNCs seemed to improve the deposition of certain extracellular matrix proteins such as type I collagen and COMP and reduce the levels of collagen type III, indicating scar tissue, compared with control horses. The response to BMMNC injection was similar to the response to cultured bone marrow–derived MSCs, although there were greater numbers of white blood cells retained in the tissue.

\textbf{Tendon-Derived MSCs}

Our work on tendon-derived stem cells is based on the rationale that cells derived from the target tissue/organ (in this case, tendon) will be phenotypically and biosynthetically more capable of stimulating tissue-specific functional repair than cells
isolated from distant sites. Tendon-derived MSCs have been isolated in humans, rats, and horses using a variety of isolation techniques, including cell migration from tendon explants, differential adherence of isolated cells, and MSC cell surface markers. In humans, it has been estimated that 1 in every 10,000 cells isolated from tendon matrix are MSCs. Tendon-derived cells isolated using these techniques can differentiate along osteogenic, adipogenic, and chondrogenic lineages. Equine tendon-derived cells have been isolated in our laboratory using a differential adherence technique developed for isolating skeletal muscle MSCs, whereby the culture medium and unattached cells are serially transferred to fresh culture flasks every 12 to 24 hours over the first 72 hours of culture. Using this technique, 17 to 19 days are required for monolayer culture expansion through 2 passages, with an average yield of $7.9 \times 10^6$ cells. Tendon-derived cells reach clinically relevant cell numbers (10 million cells) in a shorter period than bone marrow–derived cells. In addition, tendon-derived cells had higher cell viability and integrated better onto acellular tendon explants when compared with bone marrow–derived mesenchymal cells (Fig. 7) and express higher levels of tendon extracellular matrix gene mRNAs. In addition, tendon-derived cells had increased collagen and proteoglycan synthesis levels than bone marrow–derived cells. More recent studies have determined that supplementing monolayer cultures with basic fibroblast growth factor increases tendon-derived cell proliferation in monolayer, providing a means to accelerate the generation of clinically useful numbers for reimplantation.

Fig. 6. Ossification/mineralization in an SDFT 7 months after injection of neat bone marrow. Note the scattered areas of radiodensity (A; arrows) and shadowing (arrows) seen in transverse (B) and longitudinal (C) ultrasonographic images.
Efficacy
We have assessed the clinical value of tendon-derived stem cell administration in the collagenase-induced SDFT model. Horses with collagenase-induced SDFT lesions were injected with tendon-derived progenitor cells in one forelimb and an equal volume of saline in the opposite forelimb 21 days after collagenase injections. The outcome analyses from this study are still ongoing, but there were no differences in total collagen content, total proteoglycan content, or ex vivo collagen and proteoglycan synthesis between the saline-treated and the tendon-derived progenitor cell–treated groups. However, both injured tendons had significantly increased collagen I, collagen III, COMP, and tenomodulin mRNA levels when compared with normal tendon. Histologically, the extracellular matrix organization of repair tissue in tendons treated with tendon-derived cells was closer to normal tendon than the saline-treated injured tendons. Specifically, there was a significant increase in the intensity of picrosirius red staining of fibrillar collagen and decreased proteoglycan staining (by toluidine blue) at the site of repair in tendons treated with tendon-derived cells, compared with controls (Fig. 8).

Tracking studies showed that tendon-derived progenitor cells stained with DiI were localized around the immediate injection site 1 week after administration, had migrated into the lesion tissues beyond the injection track by 2 weeks (Fig. 9), and had largely dispersed from the site of administration by 4 to 6 weeks. There was only minimal cell migration from the injured SDFT to adjacent sites. The tendons treated with the tendon-derived cells also had significantly increased DNA content within the lesion site during the first 2 weeks after the injection, reflecting increased cellularity. This difference was lost at later time points.

To date, only 2 cases of clinical tendonitis have been treated with tendon-derived progenitor cells (Fig. 10) (Dr Jennifer Barrett, personal communication, 2011). Both horses returned to performance, but 1 tendon injury recurred after 18 months.

Safety
In the studies completed to date, tendon-derived cells have been isolated from the hind limb lateral digital extensor (LDE) tendons of 8 research horses using an approach more commonly used to treat stringhalt. There were no immediate complications associated with intratendinous stem cells injections after the LDE tenectomy in the donor horses; however, the longer-term consequences of LDE tenectomy on subsequent athletic performance are not known.
At this time, the results from the in vivo collagenase model study have not shown a significant enough improvement in tendon healing over other MSC sources to justify the invasiveness of an LDE tenectomy to harvest the cells. However, the morphometric analyses of matrix organization and biomechanical testing of tendon specimens from this study are still ongoing, and significant beneficial effects of tendon-derived progenitor cell administration may yet be identified.

Fig. 8. Histologic sections of healing tendons stained with picrosirius red for collagen content (upper row) and with toluidine blue for proteoglycan content (lower row). (A) Normal tendon, (B) injured tendons 4 months after treatment with tendon-derived progenitor cells, and (C) injured tendons 4 months after treatment with saline injections. Fiber alignment is improved in the cell-treated lesion (comparing [B] and [C] in both rows). Collagen stains more intensively, and proteoglycan staining is less intense in the cell-treated sections (original magnification ×25).

Fig. 9. Distribution of Dil-stained (red) tendon-derived progenitor cells after intratendinous injection. Two weeks after injection, the cells are distributed longitudinally within the healing tendon lesion and are aligned with the native tendon cells (stained blue with DAPI, original magnification ×100).
SUMMARY

The potential for stem cells to improve or cure tendon and ligament injuries is still being investigated. There is still a need to understand the mechanisms of cell lineage commitment and the principles of tendon differentiation. Studies of both ESCs and adult stem cells will be required to advance the scientific and therapeutic potential of regenerative medicine most efficiently. Several questions need to be answered to identify the optimal cell type and dose to be identified for each clinical condition. ESCs have the greatest potential for regenerative therapy for tendons and ligaments, which have limited capacity for self-repair. However, it is clear that existing equine ESC-like cells are not yet fully characterized, and current markers used to characterize pluripotency in equine cells are inadequate. The development of iPS cells represents

Fig. 10. Transverse (A, B) and longitudinal (C, D) ultrasonographic images of a large SDFT core lesion 14 days after injury (A, C) and 4 months after intralesional injection of tendon-derived stem cells. The echogenicity of the lesion has improved noticeably over this time. (Courtesy of Dr Jennifer Barrett, Marion duPont Scott Equine Medical Center, Virginia-Maryland Regional College of Veterinary Medicine, Leesburg, VA.)
a possible alternative yet to be intensively investigated before its applicability is known in clinical scenarios.57

Adult stem cells from bone marrow have so far attracted most of the scientific attention, and both experimental data and clinical experience have suggested a positive therapeutic effect. Most importantly, MSC implantation does not result in significant deleterious effects, either from the implantation process itself or from the formation of different normal or abnormal tissues within the implantation site.23 The work performed in natural disease in the horse provides more relevant efficacy and safety data to pave the way for their use for treating tendon and ligament injuries in other species, including man.

At present, most cell therapeutic techniques for horses have been autologous in nature. However, an allogenic source would provide an off-the-shelf product, which could then be given at an optimum time in the disease process rather than one governed by isolation and culture times. This would also potentially make the product cheaper. In addition, the product could be standardized more easily, although maintaining this standard throughout the multiple population doublings necessary to supply sufficient cells is a concern. Allogeneic cell sources entail the potential for increased risk of immunologic reaction, although MSCs are immunoprivileged and additionally suppress the immune response. In addition, allogenic cells could be a possible source of disease transmission, so further quality control and regulatory issues would need to be established.

REFERENCES

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