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Suspension of Bone Marrow–Derived Undifferentiated Mesenchymal Stromal Cells for Repair of Superficial Digital Flexor Tendon in Race Horses

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ABSTRACT

It has been proven that mesenchymal stromal cells (MSCs) can differentiate into tenocytes. Attempts to repair tendon lesions have been performed, mainly using scaffold carriers in experimental settings. In this article, we describe the clinical use of undifferentiated MSCs in racehorses. Significant clinical recovery was achieved in 9 of 11 horses evaluated using ultrasound analysis and their ability to return to racing. Our results show that the suspension of a small number of undifferentiated MSCs may be sufficient to repair damaged tendons without the use of scaffold support. Ultrasound scanning showed that fibers were correctly oriented. By using undifferentiated cells, no ectopic bone deposition occurred. A sufficient number of cells was recovered for therapeutic purposes in all but 1 case. We suggest that the use of autologous MSCs is a safe therapeutic method for treating incompletely (i.e., not full-thickness) damaged tendons.

INTRODUCTION

In recent years, multipotent mesenchymal stromal cells (MSCs)[1] have been extensively studied to evaluate and understand their potential for differentiating along several mesodermic and non-mesodermic cell lines. Since the seminal paper from Verfaille,[2] it has been proved that MSCs are capable of differentiating, depending on the culture medium, along several differentiation pathways without undergoing telomere reduction or acquiring chromosome mutations. In addition to morphological and immunophenotyping studies, their extensive consistent differentiation to multiple mesenchymal lineages under controlled *in vitro* conditions is considered a key method of identifying mesenchymal cells cultures.[3] Several tissues contain populations of stem cells able to renew themselves after trauma or disease. Adult bone marrow contains mesenchymal "stem" cells that possibly contribute to the regeneration of bone, cartilage, muscle, tendon, and adipose tissue. These properties have prompted several authors to evaluate their role in regenerative medicine.[4] Their application in repairing bone using autologous cells has been shown in several animal models.[5–7] Moreover, cultured MSCs may regenerate articular cartilage[8,9] and muscle damage in muscular dystrophy.[10]

With a goal of using them in possible clinical applications, several attempts to repair tendon ruptures have been reported; in rabbit models, interesting results in repairing the Achilles tendon have been achieved[11] since 1997. Histological and immunochemical studies in rabbit models have demonstrated the quality of repair,[12] even when allogeneic stem cells were employed.[13] A basic problem in tissue repair

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is in how to administer stem cells to the damaged tissue. Several questions remain: Should the cells be (partially) differentiated or committed to the cell lineage we intend to repair, or they should be kept undifferentiated to prevent loss of proliferative potential? Should cells be administered using a scaffold to resolve handling difficulties, providing a static or dynamic support to cells and possibly sustaining the oriented differentiation to the desired lineage? There is no simple answer to these questions, although many possible solutions have been suggested.

When directed to tendon defect repair, scaffolds have usually been employed because of the specific properties of such tissue with regard to resistance, tension, sharing, and elasticity. This last property has been frequently addressed using collagen-based scaffolds[14] that may be strained after cell seeding onto a pre-tensioned suture system.[15] The number of MSCs and the density of the collagen sponge play a key role in the biomechanical properties of the implant.[16,17] However, unexpected differentiation of MSCs to osteoblasts instead of the expected tenocyte lineage, thus forming ectopic bone in the tendon, may complicate the use of collagen-based scaffolds.[18] Different approaches for the use of mesenchymal cells in repairing tendons have been followed, such as the attempt to revitalize nonviable dense grafts[19] and the use of poly(L-lactide) scaffolds assembled with cell sheets.[20] Whereas there is an obvious indication for the use of scaffolds in the attempt to repair full-thickness defects of tendons or even to fabricate ligament constructs *in vitro,*[21] their *in vivo* implantation may still have unexpected results. In fact, in addition to obvious considerations concerning mechanical properties, including resistance and elasticity, scaffolds may induce host reactivity and interfere with the expected differentiation of implanted stem cells, for example, inducing ectopic bone formation.[22] Conversely, when the tendon is only damaged, we may hypothesize that the injection of MSCs could contribute to healing. In this case, we should assume that tissue microenvironment and spatial tendon organization would induce differentiation of MSCs to tenocytes and correctly orient deposition of fibers.

To assess this point, we decided to attempt to cure, as a clinical model, horses with a typical core lesion in the superficial digital flexor tendon (SDFT). This lesion is extremely disabling for racehorses and is not easily curable, if at all.

In this study, multipotent mesenchymal stromal cells were recovered from autologous bone marrow, expanded *ex vivo,* and re-injected into the lesion. Equine MSCs were morphologically characterized and their osteogenic differentiating potential evaluated. Growth kinetics are also reported. Because of the impossibility of phenotyping these cells because a lack of antibodies against equine specificity, cells are further characterized according to gene expression analysis for some matrix-related genes. Results of cell injection were evaluated clinically, using ultrasound evaluation of the tendon, and according to performance results.

METHODS

Patients

We used 11 racehorses, 4 female and 7 male, with a median age of 5 years (range 2–15), referred for lameness and, upon ultrasound examination using 7.5 to 12 MhZ linear ultrasound transducer probe, showing a typical core lesion in the SDFT. The control group included 15 racehorses, 2 female and 13 male, with a median age of 6 years (range 4–8). Horses were treated in the veterinary department using standard therapy for SDFT lesion.[23] Selection of horses to be included in the study or control group was not based on clinical differences but on the availability of the MSC procedure and on the consent of owners.

MSC isolation and cultivation

Bone marrow aspirates were performed using sternal puncture under local anesthesia with a subcutaneous injection of 5 mL of lidocaine 2%. A total of 40 mL of marrow was drawn into two 20-mL syringes containing 2500 U of heparin (Roche, Basel, Switzerland) to prevent clotting and promptly shipped to the cell culture facility. After dilution 1:1 with Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY) bone marrow was carefully layered over Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged at $400\times g$ for 20 min. Mononuclear cells (MNCs) were collected at the media–Lymphoprep interface, washed twice in HBSS, and plated in T-150 culture flasks (Corning) containing 20 mL of low-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/mL gentamicin, and 100 μg/mL amphotericin B (medium and supplements from Gibco) at two different cell densities: $4\times10^5/cm^2$ and $8\times10^5/cm^2$. Medium was changed after 48 h to remove nonadherent cells and then 2 times per week. At day 15, cells from primary culture were detached using 0.25% trypsin/ethylenediaminetetraacetic acid (Gibco) at room temperature for 1 to 2 min and re-plated, after 2 washes in HBSS, at 5×10^3 cells/cm^2. Expanded MSCs were harvested using trypsin digestion at 80% to 90% confluence, washed twice, and re-suspended in 1.5 mL of fresh autologous serum for *in situ* injection.

Frequency of colony-forming unit enumeration assay

MNCs from aspirates were plated in T-25 culture flasks and maintained as previously described. After 2 weeks, cultures were washed in HBSS, fixed in methanol for 5 min at $-20^\circ C$, and stained with methyl-violet to reveal colonies.

In vitro *osteogenic assay*

A total of 2×10^5 expanded MSCs were plated in a 25-mm Petri dish containing 1.5 mL of medium and fed

until a confluence of 70% to 80%. At this time, osteogenic induction was performed by adding 20 μM of hydrocortisone 21-hemosuccinate (Cambrex, Walkersville, MD), 10 mM β-glycerophosphate (Cambrex), and 50 μg/mL ascorbic acid (Sigma, St. Louis, MO). After 3 weeks, staining with Alizarin-S (Sigma) was performed to reveal calcium deposits.

Reverse transcriptase polymerase chain reaction analysis

Ribonucleic acid (RNA) was extracted from 5×10^5 expanded MSCs using Tri-Reagent (MRC, Cincinnati, OH) according to the manufacturer's instructions. The complementary deoxyribonucleic acid (cDNA) synthesis reaction was performed with 1 μg of total RNA in a total volume of 20 μL containing 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), 1× first-strand buffer (50 mM Tris-hydrochloric acid (HCl, pH 8.3) 75 mM potassium chloride, 3 mM magnesium chloride ($MgCl_2$)), 1 mM deoxynucleotide triphosphates (dNTPs), 32 units of RNaseOUT (Invitrogen, Carlsbad, CA), 10 mM dithiothreitol, and 5 μM random primers. The synthesis programs included an initial incubation at 37°C for 10 min followed by incubation at 42°C for 45 min. The reaction was inactivated by heating at 99°C for 3 min. Amplification was performed in a final volume of 25 μL using 1 μg of cDNA; 0.4 μmol/L of oligonucleotide primers; and 200 μmol/L each of dNTPs, $MgCl_2$ 2 mM, and 1.5 U Taq polymerase in the 1× polymerase chain reaction (PCR) buffer (67 mmol/L Tris-HCl, pH 8.8; 16 mmol/L ammonium sulphate; and 0.01% Tween 20; all reagents from Invitrogen).

Amplification was performed for 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s after initial denaturation at 94°C for 5 min. Primers used for amplification are listed in Table 1. PCR-amplified products were resolved using 2.5% agarose gel electrophoresis.

In situ *injection of expanded MSCs*

After ultrasound localization of the SDFT, cell suspension was slowly injected directly inside the core lesion using a 2.5-mL sterile syringe.

Statistical analysis

Linear regression was applied to draw logarithmic growing curves, and nonparametric statistical analysis was performed using the Mann–Whitney test for independent samples to compare age-related groups.

RESULTS

MSC isolation and cultivation

From 40 mL of bone marrow aspirates we obtained 80×10^6 to 1.5×10^9 MNCs. After 7 days of primary culture, cells grew in groups of 50 to 100, showing a typical spindle-shape morphology. After an additional week, cells formed adherent colonies that began to proliferate in 3 dimensions and showed a multilayered structure (Fig. 1A–C). At this time, cultures were detached to obtain a median of 1.6×10^6 cells (range 40×10^3–5.2×10^6). Subcultured cells grew in monolayer and reached 90% confluence in approximately 15 days to obtain 9.5×10^6 (range 0.6×10^6–31.2×10^6) cells to infusion. We observed typical logarithmic cell growth in all cases, with a median 7.5-fold expansion for each passage.

Frequency of colony forming unit enumeration assay

Frequency of colony forming units (number of colonies on total plated cells) in bone marrow samples ranged from less than $1/10^7$ to 1/26,000. Table 2 summarizes these results.

Table 1. Sequences of Primers Designed for Reverse Transcriptase Polymerase Chain Reaction Analysis and Related Amplification Product's Length

Primer	*Sequence*	*Product*
CBFA-I	Sense: 5′-CCAACCCACGAATGCACTATC-3′ Antisense: 5′-TAGTGAGTGGTGGCGGACATAC-3′	92 bp
Collagen type I	Sense: 5′-AAGGTCATGCTGGTCTTGCT-3′ Antisense: 5′-GACCCTGTTCACCTTTTCCA-3′	114 bp
Collagen type II	Sense: 5′-AGCAGCAAGAGCAAGGAGAAG-3′ Antisense: 5′-GCAGGCGTAGGAAGGTCATC-3′	130 bp
Collagen type X	Sense: 5′-CCACCGGGACCATCAGCT-3′ Antisense: 5′-CCAGGGATTCCAGGTGGTC-3′	149 pb
Osteopontin	Sense: 5′-GCCGAGGTGATAGTGTGGTT-3′ Antisense: 5′-TGAGGTGATGTCCTCGTCTG-3′	101 bp
β-Actin	Sense: 5′-CCGCGAGCACAGAGCCTC-3′ Antisense: 5′-CTCTGCACGGGCGAAGGG-3′	135 bp

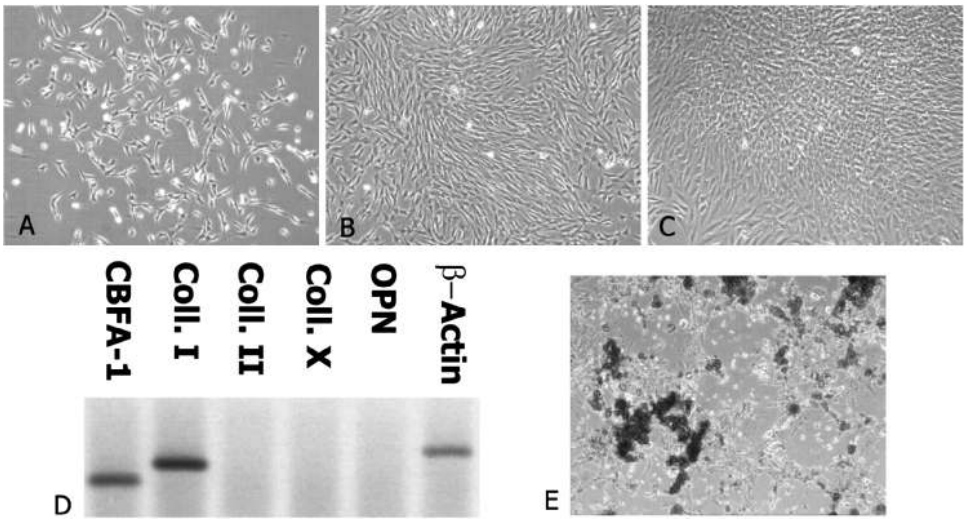

FIG. 1. Mesenchymal stem cells expanding in culture after 7 days (**A**). Cells in groups of 50 to 100 elements, after 10 days (**B**), forming colonies in monolayer. After 15 days (**C**), cells showed multilayered structures. Gel electrophoresis of reverse transcription polymerase chain reactin products (**D**). Calcium deposits in osteogenic induced cultures (**E**). Coll, collagen; OPN, osteopontin; CBFA-1, core-binding factor alpha-1.

Molecular characterization of MSCs

Expanded MSCs strongly expressed messenger RNA (mRNA) for core-binding factor alpha-1 and collagen type I as expected in an unstimulated MSC population[24] without any signs of differentiation to osteogenic or chondrogenic lineage, as revealed by lack of collagen type II or type X or osteopontin mRNA expression[25–27] (Fig. 1D). When cultured under differentiation conditions, cells showed osteogenic potential, as revealed by calcium deposition (Fig. 1E).

Clinical outcome

All 11 horses implanted with autologous MSCs exhibited no adverse reaction due to the implantation of the cells, either locally or systemically. After 10 days of stall rest, both horse groups received rehabilitation therapy consisting of 3 months of hand walk and then jogging over 2 months with increasing intensity before starting again to train and subsequently coming back to racing. Nine MSC-treated animals recovered from their clinical conditions, had an excellent ultrasound image of tendons after a period ranging from 3 to 6 months, and returned to racing with good or even optimal results in the previous category of competition in 9 to 12 months without any re-injuring event. All of them are still active more than 2 years from diagnosis. One of the 2 remaining horses received less than 1×10^6 of MSCs, and its tendon did not heal, relapsing after rehabilitatoin; the other was lost to follow-up. Ultrasound studies were repeated after

TABLE 2. CULTURE RESULTS

Patient ID	*Sex*	*Age*	*MNC* ($\times10^6$)	*CFU freq.*	*MSCs primary* ($\times10^6$)	*MSCs infused* ($\times10^6$)	*Exp. factor*	*days*
HR001	F	4	230	–	5,20	31,20	6,0	35
HR003	M	7	700	–	2,50	10,60	4,2	33
HR006	M	3	140	1/50'000	1,60	9,60	6,0	29
HR007	F	15	100	$<1/10^7$	0,04	0,84	21,0	30
HR008	F	2	140	1/26'000	4,00	19,00	4,7	23
HR009	M	10	80	1/116'000	1,90	9,60	5,0	21
HR011	M	3	560	1/420'000	1,10	9,50	8,8	18
HR012	M	13	1460	1/280'000	1,60	1,60	–	16
HR013	F	6	190	1/700'000	0,68	2,70	4,0	26
HR014	M	5	290	$<1/10^7$	0,10	0,60	6,0	29
HR015	M	3	96	1/52'000	0,60	6,30	10,5	35
Range	*4F/7M*	*2–15*	*80–1460*	*$<1/10^7$–1/26000*	*0,04–5,2*	*0,6–31,2*	*4,0–21,0*	*18–35*
Median		*5*	*190*	*1/280'000*	*1,60*	*9,50*	*7,6*	*29*

MSC, mesenchymal stem cell; CFU, colony forming unit.

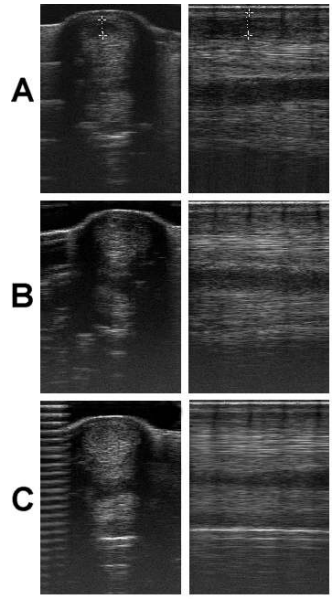

FIG. 2. Ultrasound images of core lesion in a superficial digital flexor tendon just before mesenchymal stem cell injection (**A**), 3 months after (**B**), and 6 months after (**C**) cell injection.

TABLE 3. RESULTS IN CONTROL GROUP

Patient ID	*Sex*	*Age (years)*	*Time to 2nd injuring (months)*
CTR001	M	5	6
CTR002	M	7	4
CTR003	M	7	7
CTR004	F	7	4
CTR005	M	8	6
CTR006	M	6	12
CTR007	F	4	5
CTR008	M	5	8
CTR009	M	7	12
CTR010	M	8	12
CTR011	M	6	6
CTR012	M	5	7
CTR013	M	8	12
CTR014	M	6	12
CTR015	M	5	12
Range	*2F/13M*	*4–8*	*4–12*
Median		*6*	*7*

All of the untreated horses experienced a second injuring event after the period of months reported in right column of the table.

1, 3, and 6 months. According to these studies, in 9 of 10 cases, it was possible to show initial tendon repair indicated by greater density after only 1 month. Six months after mesenchymal cell injection, tendons appeared to be almost completely repaired. Orientation of fibers revealed using ultrasound analysis was correctly parallel to the long axis of the tendon. (Fig. 2) In contrast, most of horses from the control group showed tendon ultrasound images that revealed fibrosis during the healing process (Fig. 3), and all of them were re-injured after a median time of 7 months (range 4–12) (Table 3).

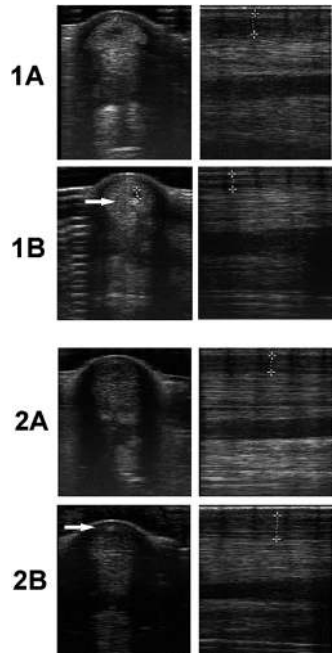

FIG. 3. Ultrasound images of two untreated cases (CTR010, CTR015) of core lesion in superficial digital flexor tendon; at day of diagnosis (**1A, 2A**) and after 3 months (**1B, 2B**). Even at first ultrasound examination is possible to localize a ultrasound-dense region due to fibrosis (white arrows).

TABLE 4. COMPARISON OF AGE-RELATED GROUPS

Patient ID	*CFU freq*	*MSCs infused ($\times 10^6$)*	*Exp. factor*	*days*
		Age < 5 years		
HR001	–	31.2	6.0	35
HR006	1/50'000	9.6	6.0	29
HR008	1/26'000	19.0	4.7	23
HR011	1/420'000	9.5	8.8	18
HR015	1/52'000	6.3	10.5	35
Median	*1/51'000*	*9.6*	*7.2*	*29*
		Age ≥ 5 years		
HR003	–	10.6	4.2	33
HR007	$<1/10^7$	0.8	21.0	30
HR009	1/116'000	9.6	5.0	21
HR012	1/280'000	1.6	–	16
HR013	1/700'000	2.7	4.0	26
HR014	$<1/10^7$	0.6	6.0	29
Median	*1/700'000*	*2.1*	*8.0*	*27.5*
	$p < 0.05$	*$p < 0.05$*	*n.s.*	*n.s.*

MSC, mesenchymal stem cell; CFU, colony forming unit.

DISCUSSION

Equine MSCs may be easily recovered and cultured from bone marrow. Their ability to grow in culture conditions does not seem to depend on the donor's age. We obtained logarithmic cell growth with similar expansion factors from a group of young horses (<5 years) and a group of horses aged 5 and older (Table 4), but the possibility of obtaining a large number of injectable MSCs seems to be related to age because of significantly different colony forming unit frequency ($p < .05$). In 10 cases, we were able to recover a sufficient number of cells, at least 1×10^6 cells, for therapeutic purposes. In only one case were fewer than 1×10^6 cells recovered, and this horse did not improve clinically. Cells were morphologically identified, and their osteogenic potential was confirmed by testing the differentiating properties.

In our experiments, equine MSCs could be expanded without sign of differentiation. This is a well-known property of mesenchymal cells,[2] but the demonstration of the absence of osteoblast differentiation in cells to be used for regenerative therapy is relevant, to avoid possible ectopic differentiation of injected cells.

The use of these cells for tendon repair has been previously suggested,[11–13] but to the best of our knowledge, there are no extensive reports about clinical applications in horse racing. Racing is stressful for the SDFT, and the return to competitive activity without relapses appears to be a significant test of functional recovery. There are no proven therapies for this pathology.[23] Horses treated with standard methods may repair lesions with phlogistic tissue. However, all of them relapse soon after restarting training or competition activities. Tendon repair has been attempted in experimental reports, mostly using scaffolds to improve tendon resistance and to handle mesenchymal cells during insertion.[14,17]

Our results show that, in patients with an incompletely (not full-thickness) damaged tendon, the scaffold may not be necessary and that excellent results, compared with typical scar-tissue images reported in follow-up of this pathology (Fig. 3), may be achieved by injecting a cell-rich solution directly in the lesion.

The tissue microenvironments appear to be able to induce cell differentiation and fiber production, as revealed uisng ultrasound scanning. The correct orientation of fibers strongly suggests that microenvironment and lines of tension and relaxation determine correct tissue morphology. The ability of tissue microenvironments to induce cell differentiation could render unnecessary a partial or total *ex vivo* differentiation. In addition to the simplification of methodology and decreased material contamination, preventing *ex vivo* differentiation appears to be a useful method for maintaining the growth potential of cells.

One relevant concern about the therapeutic use of stem cells is the possibility that injected cells may differentiate toward unexpected lines, thus causing the appearance of ectopic tissues. This possibility has been proven using cells suspended in the appropriate scaffolds to repair tendons.[18] Bone formation in the tendon to be repaired may constitute a major problem, because of reduced resistance and possible consequent tissue reactivity.

In our series, no bone deposition was shown, according to ultrasound, in the treated tendons, thus confirming our working hypothesis that direct infusion of undifferentiated MSCs could represent a safe therapeutic approach to tendon repair and suggesting that tissue is able to orient cell differentiation correctly, allowing, in parallel, the necessary *in situ* cell expansion.

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