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Multipotency of equine mesenchymal stem cells derived from synovial fluid

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ABSTRACT

Cartilage regeneration with cell therapy following arthroscopic surgery could be used in racehorses with intra-articular fractures (IAF) and osteochondritis dissecans (OCD). The aims of this study were to investigate the origin and multipotency of stromal cells in the synovial fluid (SF) of horses with intra-articular injury and synovitis, and to provide a new strategy for regeneration of lost articular cartilage. Mesenchymal stromal cells were isolated from SF of horses with IAF and OCD. Multipotency was analysed by RT-PCR for specific mRNAs and staining for production of specific extracellular matrices after induction of differentiation. The total number of SF-derived mesenchymal stromal cells reached $>1 \times 10^7$ by the fourth passage. SF-derived cells were strongly positive (>90% cells positive) for CD44, CD90 and major histocompatibility complex (MHC) class I, and moderately positive (60–80% cells positive) for CD11a/CD18, CD105 and MHC class II by flow cytometry. SF-derived cells were negative for CD34 and CD45. Under specific nutrient conditions, SF-derived cells differentiated into osteogenic, chondrogenic, adipogenic and tenogenic lineages, as indicated by the expression of specific marker genes and by the production of specific extracellular matrices. Chondrogenic induction in culture resulted in a change in cell shape to a 'stone-wall' appearance and formation of a gelatinous sheet that was intensely stained with Alcian blue. SF may be a novel source of multipotent mesenchymal stem cells with the ability to regenerate chondrocytes.

Introduction

Arthroscopic surgery to remove osteochondral fragments and to curette the surrounding degenerative cartilage has been accepted worldwide in Thoroughbred horses with intra-articular fractures (IAFs) and osteochondritis dissecans (OCD). To repair defects with hyaline cartilage, cartilage regeneration using mesenchymal stem cells (MSCs) derived from bone marrow (BM) (Arnhold et al., 2007) or adipose tissue (AT) (Braun et al., 2010) has been investigated.

The advantage of AT-MSCs is the abundance of MSCs per unit weight of tissue (Burk et al., 2013). However, we believe that a therapeutic strategy using AT-MSCs may be unacceptable in racehorse practice, because of the lower somatic fat quantities in Thoroughbred horses compared to Standardbred horses (Kearns et al., 2001). Paracentesis to aspirate BM is invasive and must be done with care to avoid contamination (Vidal et al., 2007).

Stem cells are increased in the synovial fluid (SF) of human beings with joint disease and injury (Jones et al., 2004; Morito et al., 2008). Improved clinical outcomes have been reported following treatment of cartilage deficits in human beings using MSCs derived from the synovium and SF (Nimura et al., 2008; Lee et al., 2011; Sekiya et al., 2012; Suzuki et al., 2012). If MSCs can be isolated from SF and expanded over a short time period after a racehorse is injured, they may be useful as a new strategy for cartilage regeneration. The aims of this study were to investigate the proliferative capacity, phenotypic characteristics and multipotency of cells in the SF associated with intra-articular injury and synovitis, and to provide a new strategy for regenerating lost or damaged cartilage with SF-MSCs.

Materials and methods

Samples

SF (3–4 mL per joint) was collected aseptically from the carpal, fetlock or tarsal joints of 11 Thoroughbred horses with IAF or OCD at the time of arthroscopic surgery (Table 1). SF samples were also obtained from nine diseased and nine normal Thoroughbred horse joints to compare the number of MSCs in the SF (Table 2). AT and BM were collected from two other horses (a male aged 10 years and a female aged

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Table 1
Profiles of synovial fluid (SF) samples obtained from the joints of horses with intra-articular fractures (IAFs) or osteochondritis dissecans (OCD).

Sample number	Age (years)	Sex	Disease	Diseased site (limb/joint)	Period of clinical onset (weeks)
1	3	F	IAF	RF/Carpus	2
2	2	F	IAF	RF/Fetlock	4
3	2	F	IAF	LF/Fetlock	4
4	1	F	IAF	LH/Tarsus	3
5	1	F	OCD	LH/Tarsus	3
6	3	M	IAF	RF/Carpus	2
7	3	F	IAF	RF/Carpus	2
8	3	M	IAF	RF/Carpus	2
9	3	M	IAF	RF/Carpus	2
10	6	M	IAF	RH/Tarsus	2
11	1	F	OCD	LH/Tarsus	No signs

SF samples were aseptically obtained from carpal, fetlock or tarsal joints of 11 Thoroughbred horses with IAF or OCD. M, male; F, female; RF, right forelimb; LF, left forelimb; RH, right hind limb; LH, left hind limb.

3 years) that were free of any joint diseases. All procedures were approved by the Animal Care and Use Committee of Kagoshima University (approval number A11037; date of approval 26 March 2012).

Isolation and expansion of stromal cells from synovial fluid

SF was diluted with five volumes of phosphate buffered saline (PBS), filtered through a 70 μm nylon filter (Cell Strainer, BD Falcon) to remove debris and centrifuged at 160 *g* for 5 min at room temperature. After decanting the supernatant, the pellet was resuspended and plated in a 25 cm^2 culture flask in complete culture medium (CCM) consisting of Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies), 10% fetal bovine serum (FBS, Thermo Scientific) and 1% antibiotic-antifungal preparation (100 U/mL Penicillin G, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B; Antibiotic-Antimycotic, Life Technologies). After incubation at 37 °C in 5% CO_2 for 9 days, cells adhering to the bottom of the flask were washed with PBS and harvested as described below. The medium was changed on days 4 and 7 (D7; Passage 0, P0). The number of colonies was counted at P0 in the 18 SF samples from the nine diseased and nine normal horse joints (Table 2).

Cultured cells were harvested with 0.05% trypsin and 0.2 mM ethylene diamine tetraacetic acid (Trypsin-EDTA, Life Technologies), and centrifuged. After decanting the supernatant, the pellet was rinsed with CCM and the cells were replated at 1×10^6 cells in 150 cm^2 dishes and cultured for 9 days. The medium was changed every 3 days for 9 days (P1). This serial process of passaging was repeated to obtain $>1 \times 10^7$ cells for reverse transcription (RT)-PCR and flow cytometry. The total number of cells was determined with a cell counter at every passage from P1 to determine proliferation rates, which were calculated as the cell doubling number, cell doubling time and daily duplication rate using the following formulas:

$$\text{Cell doubling number} = \ln(\text{final number of cells}/\text{initial number of cells})/\ln(2)$$

$$\text{Cell doubling time} = \text{Cell culture time}/\text{cell doubling number}$$

$$\text{Daily duplication rate} = \text{Cell doubling number}/\text{cell culture time} = 1/\text{cell doubling time}$$

Surface markers and multipotency of the cells were analysed at the fifth passage (P5). Normal SF-MSCs were analysed at P6, when sufficient numbers of cells were obtained.

Isolation and expansion of stromal cells from adipose tissue and bone marrow

Horses were sedated by IV injection with 4 μg/kg medetomidine HCl (Domitor, Zenoaq) and 10 μg/kg butorphanol tartrate (Vetorphale, Meiji Seika), and 25–30 g AT were obtained from the gluteal subcutis using liposuction. Liposuction solution (100–200 mL) consisting of physiological saline (Normal Saline, Otsuka) containing 400 μg/mL lignocaine HCl and 0.4 μg/mL adrenaline (Xylocaine injection 1% with Epinephrine, AstraZeneca) was injected SC through a 10 mm skin incision and AT was aspirated with a probe (Collection Cannula, 14 G, length 30 cm; Cytori) connected to a 50 mL syringe. This procedure was repeated 10 times. The aspirated AT was digested with a 2× volume of PBS containing 0.1% collagenase (Life Technologies) at 37 °C for 90 min, filtered through a 70 μm nylon filter (Cell Strainer, BD Falcon) and centrifuged at 160 *g* for 5 min at room temperature. The cell pellet was re-suspended in CCM and incubated at 37 °C in 5% CO_2 for 9 days, then cells adhering to the bottom of the flask were washed with PBS and harvested. The medium was changed on the day 6 (D6; P0).

BM (30–35 mL) was collected from the fifth segment of the sternum by needle core biopsy under local anaesthesia with 20 mg/mL lignocaine HCl (Xylocaine Injection 2%, AstraZeneca) and the same sedatives and analgesics as used for AT collection. A bone marrow biopsy needle (11 G, length 10.2 cm; Angiotech) was inserted through a 10 mm skin incision. BM was aspirated with a 20 mL syringe containing 5000 IU heparin, then re-suspended in CCM. After incubation at 37 °C in 5% CO_2 for 9 days, the cells adhering to the bottom of the flask were washed with

Table 2
Colonies of normal and diseased equine synovial fluid (SF)-derived mesenchymal stem cells at passage 0.

Source of stem cells	Sample number	Disease	Limb/Joint	Colonies at passage 0	Mean ± standard deviation	*P* value
SF from diseased joints	D1	IAF	RF/Carpus	73	166.9 ± 100.9	0.001
	D2	IAF	RF/Carpus	62		
	D3	IAF	LF/Carpus	94		
	D4	IAF	RF/Carpus	121		
	D5	IAF	LF/Carpus	364		
	D6	IAF	RF/Carpus	271		
	D7	OCD	RH/Tarsus	161		
	D8	IAF	RF/Carpus	136		
	D9	IAF	RF/Carpus	220		
SF from normal joints	N1	ND	LF/Carpus	10	8.3 ± 6.5	
	N2	ND	LF/Carpus	21		
	N3	ND	LF/Carpus	10		
	N4	ND	RF/Carpus	5		
	N5	ND	LF/Carpus	6		
	N6	ND	LH/Tarsus	3		
	N7	ND	RF/Carpus	5		
	N8	ND	RH/Tarsus	0		
	N9	ND	LF/Carpus	13		

SD, standard deviation; IAF, intra-articular fractures; OCD, osteochondritis dissecans; ND, no data; RF, right forelimb; LF, left forelimb; RH, right hind limb; LH, left hind limb.