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Differentiation potential of synoviocytes derived from joints with cranial cruciate ligament rupture and medial patella luxation in dogs

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ABSTRACT

The objective of this study was to assess the differentiation capability of synoviocytes derived from dogs with inflammatory joint conditions. Cranial cruciate ligament ruptured (CCLr) (n = 12) and medial patella luxated (MPL) (n = 10) knee joints of the dogs were used to collect the synovial membrane (SM). Synoviocytes were enzymatically released from the SM and analyzed by flow cytometry for specific cellular markers (CD44 and CD90) of mesenchymal stem cells (MSCs), while doing histopathology from another part of SM sections. Under specific culture conditions, synoviocytes were forced to differentiate into chondrogenesis, adipogenesis, osteogenesis and osteoclastogenesis to investigate the multipotency. Upon treatments phenotypes of cell cultures were analyzed by histopathology and by semi-quantitative reverse transcriptase polymerase chain reaction for the expression of each differentiation marker genes. Although flow cytometry showing similar MSCs populations in CCLr and MPL synovium, synovial cells derived from CCLr showed higher multipotency compared to MPL-derived samples. Further, synovial changes such as vascularity, mononuclear cell infiltration and cellular hypertrophy were more pronounced in CCLr-derived synovial tissue than in MPL. Taken together, these findings suggested that the differentiation capability of SM-derived multipotent stem cells varies with inflammatory severity occurring in different joint conditions.

1. Introduction

Inflammatory joint diseases leading to severe functional disability are characterized by abnormal synovial proliferation as well as destruction of articular cartilage and bone (Danks et al., 2002). The synovium is the central area of pathology of osteoarthritis (OA), rheumatoid arthritis (RA) (Sakkas and Platsoucas, 2007) and other inflammatory joint diseases including canine common knee injuries such as cranial cruciate ligament rupture (CCLr) and medial patella luxation (MPL) (Doom et al., 2008; Witsberger et al., 2008). The study of degenerative changes of SM during pathological conditions in the joint contributes to the understanding and establishing of therapeutic options in regenerative medicine. During the inflammatory process, the cell population of SM is predominantly composed of mononuclear cells and includes T and B lymphocytes, activated tartrate-resistant acid phosphatase positive (TRAP) macrophages, major histocompatibility complex (MHC) class II + dendritic cells and plasma cells (Lemburg et al., 2004; Muir et al., 2005). Proper understanding of cellularity in synovitis could be attained through elucidation of significant variations of microarchitecture in synovial tissue including the thickening of the intimal layer, increased vascularity, inflammatory cell infiltration of the sub synovial layer and the formation of multiple finger-like projections (villi) into the joint (Smith, 2011).

Stem cells, which are available in different organs and tissues, are capable candidates for the regeneration of tissue and organ systems. Mesenchymal stem cells (MSCs) are able to differentiate into lineages of mesenchymal tissues such as cartilage, bone, fat, and muscle (Branco De Sousa et al., 2014; De Bari et al., 2001). Isolation and characterization of MSCs from canine bone marrow (BM) have been previously described (Kisiel et al., 2012). Most of the studies have been focused on finding MSCs in different parts of the body for medical treatment. The synovial membrane is the most specialized mesenchymal tissue among joint structures which, carrying resident MSCs (Branco De Sousa et al., 2014), have the potential of multiple differentiation (De Bari et al., 2001). Synovium is the closest tissue to articular cartilage having MSCs with higher chondrogenic capacity comparing with that in other structures of joint (Mochizuki et al., 2006; Sakaguchi et al., 2005) and is the only tissue that can produce hyaline cartilage in benign conditions,

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suggesting that SM act as a source of cells for articular cartilage repair (Nagase et al., 2008).

But the differentiation ability of synoviocytes in each individual case of CCLr or MPL with erratic inflammatory severity is yet to be elucidated. To our knowledge, there are no published studies confirming an association between synovitis and differentiation ability of synoviocytes in canine patients. The objective of the study reported here was to determine whether there was a relationship between the inflammation and multipotency of the cells from SM with CCLr or MPL. We hypothesized that the dogs with CCLr or MPL, which carry different inflammatory severities, would be more likely to have a varying ability of multipotency. The findings of this study support the hypothesis and suggest that pathological changes and differentiation capability of progenitors of SM in early synovitis could vary among CCLr and MPL.

2. Material and methods

2.1. Specimens

Synovial membrane samples were collected from dogs diagnosed with ruptured cranial cruciate ligaments (n = 12) and medial patella luxation (n = 10) while undergoing surgical exploration of the affected knee joint followed by surgical joint stabilization. Apart from ligament rupture, the animals were otherwise healthy. Twenty-two skeletally-mature, mixed small breed of genders, age 4 to 7 years (4.7 ± 1.3 years) and weighing 3 to 8 kg were used for this experiment after obtaining owner consent. All dogs were evaluated at Hokkaido University Veterinary Teaching Hospital (HUVTH). The use of clinical samples and all samples from experimental dogs was in accordance with Hokkaido University Institutional Animal Care and Use Committee guidelines (approval number: 12-0059). The medical history of each affected dog including information obtained from both the owner and the referring veterinarian was documented. Tentative diagnosis of CCLr was made on the basis of results of a drawer or tibial compression test in addition to diagnosing MPL by radiography and based on palpation of an unstable knee cap. For each dog, CBC, serum biochemical profile and urinalysis were performed.

2.2. Isolation and culture of synovial cells

The synoviocytes were isolated from the synovial tissue using a modification of published protocols (Burmester et al., 1987). The synovial tissue was dissected away, washed with Ca2 +/and Mg2 + free phosphate-buffered saline (PBS) twice and finely minced. The minced tissue was digested with 0.2% collagenase type I (Wako pure chemicals, Osaka, Japan) in Dulbecco's modified eagle medium (DMEM, Invitrogen, NY, USA) containing 10% heat-inactivated fetal bovine serum [(FBS) Nichirei Bioscience INC., Tokyo, Japan] and antibiotic-antimycotic solution (100 units/ml penicillin, streptomycin and gentamycin; Wako) for 90 min at 37 °C. After filtering through the 70-mm cell strainer (Safar Co. Ltd., Osaka, Japan) the cell suspension (3 ml) was layered on 4 ml of ficoll/paque (Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 400*g* for 30 min. The interface layer was re-suspended in 4 volumes of DMEM and washed three times by centrifugation at 250*g* for 10 min. The cells were finally suspended in DMEM containing 10% FBS and seeded in (10^6 cells/well) 48-well multi-well plates (Life Sciences, Oneonta, NY, USA).

2.3. Cytometry

Confluent monolayer primary cultured cells were washed thrice with PBS and harvested by treatment with Ethylene diamine tetra acetic acid (0.05% EDTA, Dojindo institute, Tokyo, Japan) in 0.02% trypsin (Wako pure chemical, Tokyo, Japan). After centrifugation (300g), cell sediment was collected and washed with PBS in micro tube (BMbio, Tokyo, Japan). This was repeated 3 times. Aliquots of 1×10^5 cells suspension were incubated with rat anti-CD44 monoclonal antibody (clone YKIX337.8.7, AbD Serotec, Kidlington, UK), rat anti-Thy-1(CD90) monoclonal antibody (clone YKIX337.217, AbD Serotec, Kidlington, UK) and negative control, rats IgG2b Isotype (Institute of medical biological co. Ltd., Nagoya, Japan) for 30 min in dark at 4 °C.

After 3 times of washing with PBS, cell suspension was incubated with fluorescein isothiocyanate (FITC) labeled anti-rat IgG antibody (Sigma, Missouri, USA) at 4 °C in a dark place for 30 min. Sediment obtained in PBS suspension was filtrated through nylon mesh (Specimen advance science and technology, Tokyo, Japan) to get cell suspension. Flow cytometer (BD FACSVerse™, Becton Dickinson and Co. USA) was used for analyzing the expression of each of cell surface antigens.

Table 1
Primes used to polymerize the chondrocytes, adipocytes, osteoblasts and osteoclast-specific function genes.

Target gene	Sense and anti-sense (5–3)	Annealing temperature (°C)	PCR fragment length (bp)
Collagen II	CACTGCCAACGTCCAGATGA	54.54	215
	GTTTCGTGCAGCCATCCTTC	54.16	
Aggrecan	ACTTCCGCTGGTCAGATGGA	54.98	111
	TCTCGTGCCAGATCATCACC	53.75	
PPARG	ATCAAGCCATTCACCACCGT	59.9	149
	GCAGGCTCCACTTTGATTGC	60.1	
Osteopontin	ACGATGTAGATGAAGATGATGG	59.8	548
	GCTTTGACTTAATTGGCTGAC	60.1	
Carbonic anhydrase II	AAGGAGCCCATCAGCGTTAG	59.82	104
	GGGCGCCAGTTATCCATCAT	60.25	
MMP9	GGCAAATTCCAGACCTTTGA	56.4	166
	TACACGCGAGTGAAGGTGAG	53.4	
RANK	CCCTGGACCAACTGTAGCAT	58.7	239
	ACCCAGTGCCACAAATTAGC	55.9	
Cathepsin K	ACCCATATGTGGGACAGGAT	57.79	169
	TGGAAAGAGGTCAGGCTTGC	60.25	
GAPDH	CTGAACGGGAAGCTCACTGG	54.92	129
	CGATGCCTGCTTCACTACCT	54.03	

2.4. In vitro chondrogenesis

The *in vitro* chondrogenesis assay was performed as described elsewhere (De Bari et al., 2001). Briefly, micromass cultures were obtained by pipetting 20-µl droplets of cell suspension from SM derived monolayer culture after trypsin treatment into individual wells of 48-well plates. The cells were allowed to attach without medium at 37 °C and then chemically defined serum-free medium was added after 3 h (Harrison et al., 1991). On the next day, 20 µg/ml dexamethasone, 10 ng/ml transforming growth factor (TGF)-β and 50 M ascorbic acid (all from Sigma, St Louis, USA) were added to the medium while adding identical amount of DMEM to parallel cultures as a control for treatment. After 21 days, media was removed from culture vessel and rinsed once with PBS to fixed cells with 4% formaldehyde solution (Kanto Chemical, Tokyo, Japan) for 30 min. After fixation, wells were rinsed with PBS and stained with 1% alcian blue 8 GS (Sigma) in 0.1 N HCl for 30 min. The wells were rinsed three times with 0.1 N HCl and added distilled water to neutralize the acidity.

2.5. In vitro adipogenesis

The *in vitro* adipogenesis assay was performed as described in another place (Kisiel et al., 2012). Briefly, SM-derived cells were cultured additional 5 days after 80% cell confluence. Then adipogenic differentiation medium which was consisting of growth medium supplemented with 1 mM dexamethasone, 0.5 mM methyl-isobutylxanthine, 10 mg/ml insulin, and 100 mM indomethacin (all from Sigma) was added to the wells. After 72 h, the medium was changed to adipogenic