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Engraftment of autologous bone marrow cells into the injured cranial cruciate ligament in dogs

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ABSTRACT

Current research indicates that exogenous stem cells may accelerate reparative processes in joint disease but, no previous studies have evaluated whether bone marrow cells (BMCs) target the injured cranial cruciate ligament (CCL) in dogs. The objective of this study was to investigate engraftment of BMCs following intra-articular injection in dogs with spontaneous CCL injury. Autologous PKH26-labelled BMCs were injected into the stifle joint of eight client-owned dogs with CCL rupture. The effects of PKH26 staining on cell viability and PKH26 fluorescence intensity were analysed in vitro using a MTT assay and flow cytometry. Labelled BMCs in injured CCL tissue were identified using fluorescence microscopy of biopsies harvested 3 and 13 days after intra-articular BMC injection.

The intensity of PKH26 fluorescence declines with cell division but was still detectable after 16 days. Labelling with PKH26 had no detectable effect on cell viability or proliferation. Only rare PKH26-positive cells were present in biopsies of the injured CCL in 3/7 dogs and in synovial fluid in 1/7 dogs. No differences in transforming growth factor-β1, and interleukin-6 before and after BMC treatment were found and no clinical complications were noted during a 1 year follow-up period. In conclusion, BMCs were shown to engraft to the injured CCL in dogs when injected into the articular cavity. Intra-articular application of PKH26-labelled cultured mesenchymal stem cells is likely to result in higher numbers of engrafted cells that can be tracked using this method in a clinical setting.

Introduction

The cranial cruciate ligament (CCL) is essential for stifle joint stability and its rupture leads to functional impairment, meniscal lesions and early onset of osteoarthritis (Arnoczky and Marshall, 1977; Korvick et al., 1994). In dogs, CCL injury is common and has been treated using a variety of different surgical techniques since 1952 (Paatsama, 1952), but no single treatment option has been shown to be clearly superior. The goals of most reported techniques are to alleviate pain, decrease instability and minimize osteoarthritis (OA) (Moore and Read, 1995), but residual lameness is frequent and OA is a common sequel (Elkins et al., 1991; Innes et al., 2004; Rayward et al., 2004). The lack of ideal outcome following surgery has prompted interest in exploring new adjunctive treatment options, such as regenerative stem cell therapy. Knowledge of the benefits of these treatment strategies would be useful both for the treatment of spontaneous canine CCL injury and for investigations into degenerative anterior cruciate ligament (ACL) disease in humans using the dog as a model.

Mesenchymal stem cell (MSC) therapy is a newly developing therapeutic approach in OA that has proven useful in cartilage repair in a variety of animal models (Murphy et al., 2003; Black et al., 2007, 2008; Chong et al., 2007; Koga et al., 2008; Khan et al., 2010; Mokbel et al., 2011a, 2011b; Guercio et al., 2012; Jorgensen and Noel, 2012; Kirkby and Lewis, 2012). Bone marrow-derived MSCs hold particular promise for tissue repair because of their ability to engraft into tissues and differentiate into the target tissue cell type, including fibroblasts, osteocytes, adipocytes, chondrocytes and myocytes (Pittenger et al., 1999; Chamberlain et al., 2007). In recent studies, MSCs were found to accelerate healing of transected ligaments in animal models (Agung et al., 2006; Kanaya et al., 2007; Kim et al., 2011) and evidence suggests that fresh whole bone marrow cells (BMCs) may have superior effects compared to purified MSCs, presumably because of an additional benefit of haematopoietic stem cells (Oe et al., 2011). Indeed, injured rat ACLs treated with BMCs had more mature fibroblasts and tighter collagen bundles compared to those treated with MSCs, leading the authors to conclude that bone marrow (BM) transplantation is an effective treatment for ACL injury (Oe et al., 2011). To date, no studies have investigated the potential benefit of stem cell adjunctive treatment in dogs with experimental or spontaneous CCL injury.

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The purpose of the present study was to assess the engraftment potential of autologous BMCs injected into the articular cavity in dogs with partial or complete CCL rupture and to determine whether PKH26 red fluorescent labelling is a safe and effective way to track canine BMCs.

Material and methods

Animals

Client-owned dogs presented for surgical treatment of spontaneous partial or complete CCL rupture to the Division of Small Animal Surgery and Orthopaedics of the University of Bern were considered for inclusion in the study (see Appendix: Supplementary material "Patient data"). Informed client consent was obtained for each dog. Study inclusion criteria were diagnosis of partial or complete CCL rupture confirmed by arthroscopy and unremarkable results of routine haematological and serum biochemical analyses. Dogs were excluded if there was a recent history of illness other than pelvic limb lameness or if they had undergone previous intra-articular application of any substance or previous surgery on the affected limb. Dogs were screened using an orthopaedic examination with various parameters: lameness, functional disability, range of motion and pain on manipulation. For each parameter a score was recorded at first time of presentation and 3 months after surgery. The scores assigned were based on a 4-point scale, 0 (best) to 4 (worst) (for details see Appendix: Supplementary material).

All animal experiments were reviewed and approved by the Commission of Animal Experimentation of the Canton of Bern, Switzerland (BE42/12; date of approval, 30 April 2012).

Isolation of bone marrow cells

Bone marrow was harvested from the proximal humerus in each dog using a 13-G BM biopsy needle connected to a 10-mL syringe containing 2 mL of heparin solution (3000 U/mL). A total of 15 mL BM was aspirated and immediately injected into a transfer bag containing 7 mL citrate phosphate dextrose adenine solution. The BM aspirate was passed through a blood transfer filter set into a 20-mL syringe, and cells were separated by density gradient centrifugation at 445 *g* for 35 min. The interface with the nucleated cell fraction was transferred and washed twice in phosphate buffered saline (PBS) before counting and partitioning for PKH26 labelling, intra-articular injection, and cultivation (see Appendix: Supplementary material for details).

Flow cytometric characterization of cells

Freshly isolated cells and cultured cells were evaluated by flow cytometry for the specific MSC markers, CD90 (YKIX337.217, eBioscience) and CD44 (FAB5449A, R&D), and for the haematopoietic stem cell marker, CD45 (YKIX716.13, eBioscience). Data were analysed using a flow cytometer (LSR II, BD Bioscience) and commercial software (FACSDiva, BD Bioscience).

PKH26 labelling

Labelling of cell membranes was performed using the PKH26 Red Fluorescence Kit (Sigma-Aldrich) according to the manufacturer's instructions. After staining, a portion of PKH26-BMCs was resuspended in PBS at a concentration of 1×10^7 cells/mL for intra-articular injection. In addition, stained cells were suspended in complete medium for evaluation of dye cytotoxicity, growth characteristics and fluorescence intensity.

Evaluation of PKH26 cytotoxicity

The effect of PKH26 labelling on cell viability was tested using a colorimetric MTT assay. For this, MSCs from the second passage were stained with PKH26 dye at 2×10^{-6} M/10^6 cells and 4×10^{-6} M/10^6 cells. The assay was conducted in replicate with MSCs from four dogs as described elsewhere (Waldherr et al., 2012). Cell viability in each well, measured as the optical density (OD), was calculated as follows: 100 × (OD of sample well – OD of blank well)/(OD of control well – OD of blank well). Mean values of repeated measurements were used for analysis.

Population doubling time

Growth characteristics of MSCs at the first and second passages unstained and stained with PKH26 (2×10^{-6} M and 4×10^{-6} M) from six dogs were investigated. The MSCs were seeded into a 24-well plate at a density of 2.1×10^3 cells/cm^2. After a recovery time of 48 h, three wells were detached daily for a period of 8 days and cell numbers were counted in a haemocytometer. The population doubling time was computed using an online calculator.[1]

[1] See: http://www.doubling-time.com/compute.php (accessed 15 August 2014).

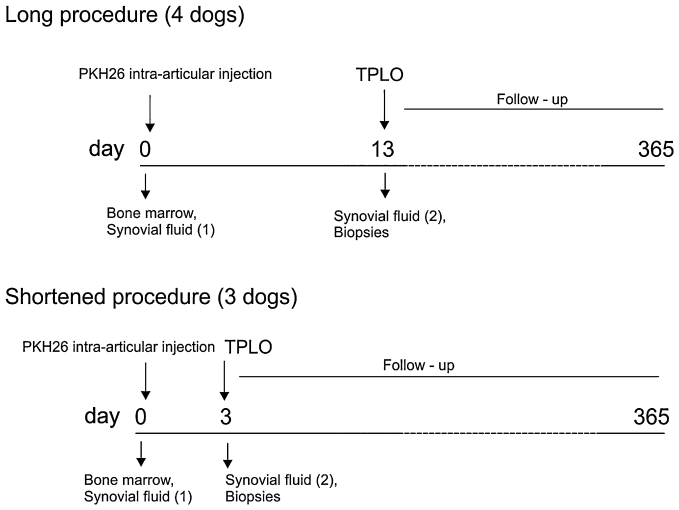

Fig. 1. Timetable of the study design. Group 1 underwent the long procedure, group 2 the shortened procedure. TPLO, tibial plateau levelling osteotomy.

PKH26 fluorescence intensity

Fluorescence intensity was assessed in freshly isolated BMCs and MSCs during cell proliferation over 16 days as described in detail in the Appendix: Supplementary material.

In vivo experimental protocol

The experimental schedule is summarized in Fig. 1. On day 0, dogs presenting with signs of CCL injury were clinically examined. BM was harvested and preoperative radiographs were performed under general anaesthesia. BMCs were isolated and labelled with PKH26 (final concentration: 2×10^{-6} M PKH26 and 1×10^7 cells/mL) within 3 h of harvesting. Synovial fluid was first aspirated and a total of 1×10^7 PKH26-labelled BMCs diluted in 1 mL PBS was injected immediately afterwards through the same needle under aseptic conditions. An aliquot of remaining BMCs was used for microbiological quality control. The dogs were then presented again for stifle arthroscopy and tibial plateau levelling osteotomy (TPLO) either 13 days (Group 1) or 3 days (Group 2) following intra-articular BMC injection. Prior to arthroscopy, synovial fluid was again collected.

Tissue collection

During arthroscopy immediately prior to surgical treatment by TPLO, the gross appearance of the stifle joint was evaluated and biopsies of the damaged CCL and synovial membrane were excised. Synovial membrane was harvested craniomedially and craniolaterally to the optic port that was located lateral to the patellar ligament halfway between patella and tibial tuberosity. Tissues were snap frozen on dry ice in O.C.T. compound (Tissue-Tek). Each block was cut into 5 µm sections at 10 µm intervals and placed on specimen slides. Sections were stored at –80 °C pending fluorescence microscopy.

Fluorescence microscopy of harvested samples

Slides were examined for PKH26 fluorescence using a confocal laser scanning fluorescence microscope (FluoView FV1000, Olympus) after counterstaining with TOTO-3 iodide (Life Technologies). Sections were defined as positive if a clear cell structure with spindle-shaped fibroblast-like morphology was detected showing at least partial red fluorescence in the membrane and far red fluorescence of the nucleus. Synovial fluid samples were examined for PKH26 fluorescence after centrifugation in a 96-well plate.

Synovial fluid analyses

Because of a possible effect of BMCs on cytokine production and immune cell attraction, synovial fluid obtained before and after intra-articular BM injection was examined cytologically, and transforming growth factor (TGF)-β1 and interleukin (IL)-6 were quantified using a commercial ELISA (canine TGF-β1, IL-6 Quantikine ELISA Kit, R&D) according to the manufacturer's protocol.