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Evaluation of cartilage, synovium and adipose tissue as cellular sources for osteochondral repair

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ABSTRACT

Osteochondral lesions are a major cause of pain and disability in several species including dogs, horses and human beings. The objective of this study was to assess three potential sources of canine cells for their osteochondral regenerative potential. Cartilage, synovium and adipose tissue cells were grown in pellet culture in chondrogenic or osteogenic media. Cartilage-derived pellets displayed the best chondrogenic differentiation as indicated by significantly higher COL2A1 and SOX9 mRNA expression, greater glycosaminoglycan content, and higher retention of Safranin-O stain compared to the synovium and adipose-derived cells.

Following application of the osteogenic media, all three cell sources exhibited small areas of positive alizarin red staining. Poor intracellular alkaline phosphatase activity was found in all three cell types when stimulated although osteocalcin and RUNX2 expression were significantly increased. Cells isolated and cultured from canine articular cartilage retained their specific chondrocytic phenotype. Furthermore, canine adipocytes and synovial cells did not undergo chondrogenic differentiation and did not exhibit evidence of multipotency. Although osteogenic differentiation was initiated at a genomic level, phenotypic osteoblastic differentiation was not observed. The findings of this study suggest that cells isolated from canine adipose tissue and synovium are sub-optimal substitutes for chondrocytes when engineering articular cartilage in vitro.

Introduction

Osteochondral lesions are a major cause of pain and disability in many species, and there is active research into 'regenerative treatments' for conditions such as osteochondritis dissecans (OCD) and osteoarthritis (Magnussen et al., 2009). Current methods for the treatment of articular cartilage defects, while demonstrating some benefits, have been variable (Derrett et al., 2005; Vasiliadis and Wasiak, 2010). Tissue-engineering techniques could potentially facilitate both chondral and osteochondral repair, and cells exhibiting multipotentreparative properties have been identified in adult tissue such as adipose tissue (Frohlich et al., 1972; Zuk, 2001) and synovium (De Bari et al., 2001).

The dog is of particular interest in this context as, not only does osteochondral pathology occur naturally (Person, 1989; Fitzpatrick et al., 2009), but this species is often studied in translational research (Ahern et al., 2009; Chu et al., 2010). Although the differentiation potential of bone marrow-derived mesenchymal stem cells from adult dogs have been studied in vitro (Kadiyala et al., 1997), there has been less research into the potency of stromal cells derived from other adult canine tissues. This is particularly important, given the growing clinical application of canine stromal cell therapy for diseases such as osteoarthritis (Black et al., 2007, 2008). The objective of this study was to investigate the chondrogenic and osteogenic differentiation potential of cells taken from canine cartilage, synovium and adipose tissue with a view to their future potential in tissue engineering therapy for osteochondral defects.

Materials and methods

Tissue sampling

Samples of cartilage, synovium and adipose tissue (infra-patellar fat pad) were harvested in an aseptic manner, with owner consent, from grossly normal stifles of six dogs euthanased for reasons unrelated to this study. All dogs were skeletally mature neutered males aged between 4 and 7 years old. Samples were placed in Dulbecco's Modified Eagles Medium (DMEM) with 5% fetal calf serum (FCS) containing 100 units/mL of penicillin and 100 units/mL of streptomycin (all from Cambrex).

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Isolation of cells from cartilage, synovium and adipose tissue

Tissue samples were digested overnight at 37 °C in 0.1% (w/v) collagenase type II (Worthington Biochemical Corporation) in DMEM with 5% FCS, 100 units/mL of penicillin and 100 units/mL of streptomycin. To isolate stromal cells from the infra-patellar fat pad, the tissue was digested for approximately 5 h at 37 °C in 0.2% (w/v) collagenase type I (Worthington Biochemical Corporation) in DMEM supplemented as previously. Subsequent to digestion, cells were processed similarly, as described below.

For primary culture, cells were filtered through a 40 μm cell strainer, washed and then re-suspended in standard growth media (DMEM supplemented as previously), counted and seeded at a density of 10^4 cells/cm^2 in tissue culture flasks. Monolayer expansion was in standard growth media at 37 °C in a humidified atmosphere containing 5% CO_2. At confluence, the cells were detached from the tissue culture plastic using 0.05% trypsin–EDTA (Cambrex), and re-seeded at 5×10^3 - cells/cm^2. Subsequently, confluent cultures were harvested by trypsinisation as described and used in the differentiation studies detailed below.

Chondrogenic differentiation

A pellet culture system was used to assess the chondrogenic potential of the cells from all three tissue sources (Solursh, 1991; Zhang et al., 2004). Cell suspensions harvested by trypsinisation were centrifuged at 200 *g* for 4 min and re-suspended in sufficient chondrogenic media (serum-free DMEM supplemented with antibiotics as before, but with additional 10 μg/mL ITS+1 liquid media supplement, 10 nM dexamethasone, and 25 μg/mL ascorbic acid [all from Sigma], and 10 ng/mL transforming growth factor-β3 [R and D Systems]) to create a cell density of 500,000 cells/mL of media.

For each pellet, 1 mL of the cell suspension was added to a 15 mL conical polypropylene tube and centrifuged at 200 *g* for 4 min. A total of six cell pellets were made from each cell type from each dog. Two pellets were used for gene expression, two for biochemical, and two for histological, analysis. The pellets were incubated, with loosened lids, for 14 days at 37 °C in 5% CO_2. The medium was changed every 2 days and at the completion of the culture period, the wet weights of the pellets were recorded.

Osteogenic differentiation

Cells from all tissue sources, maintained in a monolayer culture and harvested as described above were re-seeded at a density of 5×10^3 cells/cm^2 in six-well culture plates and grown to 70% – 80% confluence in standard medium at 37 °C in 5% CO_2. At this point the media was replaced with osteogenic medium (DMEM containing 10% FCS, 100 units/mL penicillin, 100 units/mL streptomycin, 10 mM β-glycerophosphate, 10 nM dexamethasone, and 0.1 mM ascorbic acid) (Rodriguez et al., 2004). Control cells were cultured in standard medium throughout. All cells were subsequently cultured for 14 days at 37 °C in 5% CO_2 with twice weekly medium changes.

Gene expression analysis

For chondrogenic cultures, at the end of the culture period, pellets were disrupted using molecular grinding resin (Geno Technology) and the total RNA was prepared using tri-reagent (Sigma) according to the manufacturer's protocol. The RNA was quantified and cDNA produced using 1 μg of total RNA/sample, via a standard reverse transcription (RT) reaction using random hexamer primers and the MMLV (Moloney murine leukaemia virus) RT enzyme (Promega). To determine the level of chondrogenic induction, collagen I (type I collagen, alpha 2 chain [COL1A2]), collagen II (type II collagen, alpha 1 chain [COL2A1]), the extracellular matrix proteoglycan aggrecan (AGG), and the transcription factor SOX9 mRNA expression were each quantified by quantitative (q)RT-PCR using primers as previously described (Clements et al., 2006), or as listed in Table 1.

The experiments were performed on an ABI 7300 qRT-PCR machine using ABI SYBR green qRT-PCR master mix kits (ABI). To assess osteogenic gene expression, qRT-PCR was used to measure mRNA concentrations of the bone-specific osteocalcin (OC), secreted phosphoprotein 1 (SPP1), and the runt-related transcription factor-2 (RUNX2) genes using primers listed in Table 1. In all assays, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous reference gene.

Biochemical analyses

Chondrogenic cell pellets were digested in 10 units/mL of papain (Sigma) in 0.1 M sodium acetate, 5 mM L-cysteine, 2.4 mM EDTA, at pH 5.8 overnight at 60 °C. The papain digests were used to: determine the cellularity of the pellets using a DNA assay (Quant-IT PicoGreen dsDNA stain, Invitrogen); and quantify glycosaminoglycan (GAG) deposition in the pellet extracellular matrix (ECM) using the 1,9-dimethylmethylene blue (DMMB) assay. The absorbance values at 570 nm were obtained and shark chondroitin sulphate (Sigma) standards were used to generate a standard curve. For osteogenic cultures, alkaline phosphatase activity from cell lysates was analysed by measuring the colourimetric hydrolysis of *p*-nitrophenol phosphate into *p*-nitrophenol, read at a wavelength of 405 nm. A total of 15 μg of protein was used for each analysis: this was previously calculated by a standard protein assay at 595 nm (Bio-Rad Protein Assay Kit, Bio-Rad).

Histological and immunohistochemical analyses of cell pellets

Chondrogenic cell pellets were fixed in 4% formaldehyde for 2 h, dehydrated and embedded in paraffin wax. Tissue sections, 5 μm thick, were de-waxed and stained with 0.1% Safranin-O (Sigma) to facilitate proteoglycan detection within the ECM. For immunohistochemical analysis, de-waxed sections were pre-digested with chondroitinase ABC (Sigma), and non-specific binding was eliminated by incubation with blocking solution (10% donkey serum [Sigma] and 1% bovine serum albumin [BSA] in PBS). Slides were incubated overnight with antibodies against collagen type I (C-18) and II (N-19) diluted 1:100 in blocking solution. Subsequent incubations with a biotin-conjugated donkey anti-goat secondary antibody (all antibodies from Santa Cruz Biotechnology), and a streptavidin–horseradish peroxidise labelling kit (with 3,3′-diaminobenzidine, [Sigma]) were used to visualise antibody–antigen conjugation.

As a negative control, blocking solution was used in place of the primary antibodies. Light microscopic images were recorded using an Axiovision light microscope (Carl Zeiss MicroImaging) and camera system. In osteogenic differentiation cultures, mineralisation within the monolayer cultures was assessed by staining with either 1% (w/v) alizarin red or 1% (w/v) silver nitrate (both from Sigma). In all cases, staining was graded semi-quantitatively as 'negative', 'mild', 'moderate' or 'strong'.

Statistical analyses

Linear mixed effects models were used to investigate differences in gene expression under different culture conditions for the three different cell sources. Significance was set at $P < 0.05$.

Results

Following adherence to the tissue culture plastic, chondrocytes lost their 'rounded' morphology and, as demonstrated previously with chondrocytes isolated from other species, proceeded to become fibroblastic in appearance. The synovial and adipose stromal cells also appeared fibroblastic in monolayer culture. All three cell types continued to proliferate at a steady rate.

Table 1
Primer sequences used for listed genes in quantitative RT-PCR analysis.

Gene	Forward	Reverse
COL1A2	CTATCAATGGTGGTACCCAGTTT	TGTTTTGAGAGGCATGGTTG
COL2A1	CTGGTGAACCTGGACGAGAG	ACCACGATCACCCTTGACTC
AGG	GGGACCTGTGTGAGATCGAC	GTAACAGTGGCCCTGGAACT
SOX-9	AAGCTCTGGAGGCTGCTGAA	ACTTGTAATCCGGGTGGTCTTTC
SPP1	TGATTTTCCCACTGACATTCCA	CCTCGGCCATCATATGAACCT
Osteocalcin	TGGAGCCCAAGAGGGAAGTA	GCTCTAGACCGGGCCATAGAA
RUNX2	ACTGTCATGGCGGGTAACG	ACCTTGCTACTTGGTTTTTCATAACA
GAPDH	CTGGGGCTCACTTGAAAGG	CAAACATGGGGGCATCAG

COL1A1, type I collagen, alpha 2 chain; COL2A1, type II collagen, alpha 1 chain; AGG, extra cellular matrix proteoglycan aggrecan; SOX-9, SOX-9 transcription factor; SPP1, secreted phosphoprotein 1; RUNX2, runt-related transcription factor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.