The knee joint loose body as a source of viable autologous human chondrocytes

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Abstract

Loose bodies are fragments of cartilage or bone present in the synovial fluid. In the present study we assessed if loose bodies could be used as a source of autologous human chondrocytes for experimental purposes. Histological examination of loose bodies and differential enzymatic digestions were undertaken, the isolated cells were cultured in alginate bead microspheres and immunolocalisations were undertaken for chondrogenic markers such as aggrecan, and type II collagen. Isolated loose body cells had high viability (≥90% viable), expressed chondrogenic markers (aggrecan, type II collagen) but no type I collagen. Loose bodies may be a useful source of autologous chondrocytes of high viability.

Introduction

Loose bodies (also known as rice bodies and knee joint mice) are fragments of cartilage or bone that float freely within the synovial fluid component of the knee joint. They may occur in single or multiple forms1 but generally only affect a single knee. Loose bodies are classified as either stable or unstable. The former are located in fixed positions in the knee and are generally well tolerated and asymptomatic while the latter are free to move around the knee joint and may be the cause of pain, knee joint swelling, joint weakness and may cause the knee to lock abruptly. Loose bodies have a traumatic origin such as dislocation of the patella2 or a complication arising from an orthopaedic procedure3 and their occurrence is more likely in patients affected by osteoarthritis (OA) or rheumatoid arthritis (RA). Smooth bodies are classified as fibrous, cartilaginois and osteocartilaginous. Fibrinous loose bodies result from intra-articular bleeding or by death of synovial tissue associated with tuberculosis, OA or RA. Cartilaginous loose bodies are caused by traumatic injury to the OA joint. Osteocartilaginous loose bodies are caused by fractures, osteochondritis dissecans.4,5 inflammation, synovial chondromatosis6 or tumours (osteochondromas).4,5 Loose bodies are normally small,6 but cases of giant loose bodies have also been reported.8 In the present study histological examination of loose bodies showed to our surprise that they were highly cellular containing large numbers of viable chondrocytes and suggested that they may be a potential source of autologous human chondrocytes for repair strategies. We subsequently went on to isolate this chondrocyte population, they displayed high viability and were highly proliferative compared to chondrocytes isolated from residual femoral and epiphyseal cartilage from the same knee joint replacements. The loose body chondrocytes expressed type II collagen, aggrecan and 5D4 KS in 3D alginate bead culture but no detectable type I collagen.

Materials and Methods

All chemicals and supplier details are as previously indicated described.10-11 Monoclonal antibodies to aggrecan (clone 4D11 2A9), type I collagen (clone I-8H5), type II collagen (clone II-4CII), biotinylated anti-mouse IgG and antirabbit IgG secondary antibody, horseradish peroxidase conjugated streptavidin have been described previously.10,12

Tissues and cells

Loose bodies harvested from total knee replacement surgical discards from 6 patients (median age 56 years, 3 male, 3 female) of the Orthopaedics Clinic at North Shore Private Hospital, St. Leonards, were obtained with informed consent under ethical approval of the human ethics and care committee of the Royal North Shore Hospital who approved all procedures.

Isolation and culture of chondrocytes from the loose bodies

Chondrocytes were enzymatically isolated from loose bodies by sequential digestion with: i) 0.1% (w/v) pronase in DMEM-F12 media containing 10% (v/v) FCS for 2 h at 37ºC; ii) 0.05% (w/v) chloridase collagenase in media for 4 h; and iii) overnight digestion with collagenase.11 The cells were spun down (10 min x 800 g) and cell viability and numbers determined on a haemocytometer using trypan blue exclusion. Examination of the front and side scatter characteristics of these cells by flow-cytometry was similar to results obtained earlier with only one major cell population evident.11 The cells were established in monolayer culture in 75 cm2 canted neck flasks at a density of 100,000 cells/mL in DMEM-F12 + 10% FCS + antibiotics under an atmosphere of 5% CO2 and 98% humidity at 37°C.11 After cellular attachment overnight, the flasks were rinsed in PBS to remove non-adherent cells and pellet (800 g x 10 min) then washed in...
dispersed at a density of $3 \times 10^6$ cells/mL alginate in sterile isotonic saline. This mixture was loaded into a 2 mL syringe and extruded drop-wise through a 23 guage needle into an agitated solution of sterile CaCl$_2$ (102 mM) in a laminar flow hood to maintain sterility. After 10 min curing time the calcium alginate beads (~10 mL/30,000 cells) were established in culture in small petri dishes (100 beads/plate) in DMEM-F12 + 10% FCS + 50 µg/mL ascorbic acid (5 mL media/plate). The plates were incubated at 37°C, in an atmosphere of 5% CO$_2$ in air, with a humidity of 98% and the medium replenished every 48 h. The loose body cells were cultured up to 4 weeks and samples of beads collected after 2, 3 and 4 weeks of culture as indicated earlier. The remaining beads were rinsed in isotonic saline and solubilized in 55 mM trisodium citrate in 150 mM NaCl (2 mL/50 beads) and the cells spun down at 800g x 10 min. The cells were then either cryopreserved in liquid nitrogen or were re-established in monolayer culture.

**Histological processing of alginate beads**

Beads were fixed 3 h in 10% (v/v) formalin, 85% (v/v) ethanol, 5% (v/v) acetic acid transferred overnight into 70% (v/v) ethanol and embedded in paraffin then sectioned at 4 µm thickness and attached to SuperFrost ultraPlus positively charged microscope slides. The slides were de-paraffinised in xylene (2 changes x 5 min), re-hydrated through graded ethanol washes (100-70% v/v) to water.

**Histochemistry**

Bead and loose body sections (4 µm) were stained for 10 min with 0.04% (w/v) toluidine blue in 0.1 M sodium acetate buffer, pH 4.0 followed by a 2-min counterstain in 0.1% (w/v) fast green FCF. Sections were also stained in Mayers Haematoxylin (5 min), rinsed in tap water blued in Scotts Blueing solution (1 min) and counterstained in 0.0001% (w/v) eosin (5 min).

**Immunolocalisation of type I and type II collagen, aggrecan and keratan sulphate**

Endogenous peroxidase was blocked in bead samples with 0.3% (v/v) H$_2$O$_2$ for 10 min then blocking undertaken in DAKO non-protein blocking agent. Aggrecan immunolocalisation were pre-digested with chondroitinase ABC (0.1 U/ml) in 50 mM Tris HCl pH 7.2 + 2% (v/v) BSA for 1 h, type I and II collagen immunolocalisations were pre-digested with proteinase K for 6 min and bovine testicular hyaluronidase (1000 U/ml) for 1 h at 37°C in phosphate buffer pH 5.0. The bead sections were incubated with anti-aggrecan (1/10,000 dilution), anti-type I collagen (1/500 dilution), anti-type II collagen (1/200 dilution) and MAb 5-D-4, anti-KS (1/1000 dilution) in TBS + 2% (w/v) BSA overnight at 4°C then biotinylated anti-mouse and anti-rabbit IgG antibodies and horse-radish peroxidase conjugated streptavidin were added using Nova RED substrate for visualisation. Negative control sections were run omitting primary Ab or using an irrelevant primary antibody. Both yielded negative results. The stained specimens were examined using a Leica photomicroscope linked to a DFC 480 digital camera using bright-field illumination.

**Results**

Loose bodies were observed in 12 of 18 total knee joint replacements, and typically 2-5 mm in size, smooth and glistening. Histological examination demonstrated a high cell density and abundant deposition of proteoglycan in the loose bodies (Figure 1). The loose body contained a central necrotic core where the cells were arranged in clumps. Closer inspection within the loose body sections revealed a tran-
sition in cell morphology and tissue organisation from the surface zones into the transitional zone through to the cartilaginous and necrotic core (Figure 1a-d). The surface zone region contained a population of flattened fusiform cells in a fibrous matrix overlying a mixed population of cells of a rounded chondrocyte like morphology (Figure 2a). This led into the transitional zone where larger chondrocyte-like cells were also evident (Figure 2b). The cartilaginous zone contained closely packed large chondrocyte-like cells (Figure 2c). The central core contained clumps of cells, many were dead or necrotic and many of the lacunae were unoccupied in the tissue sections examined (Figure 2d). Examination of surface zone 1 revealed stratification of the cells into three discernable zones, two surface zone regions containing cells of a flattened morphology but little proteoglycan (Figure 2f), and a cartilaginous zone underlying this containing a dense population of rounded chondrocyte like cells surrounding by metachromatically stained proteoglycan (Figure 2g). Surface zone 2 displayed similar traits to surface zone 1 with a surface fibrous region containing flattened cells overlying a cartilaginous zone containing larger chondrocytes in a proteoglycan rich matrix (Figure 2h,i).

Examination of the front and side scatter characteristics of the chondrocytes released from the loose bodies by flow-cytometry showed only one cell population (Figure 3a). A small proportion of dead/non-viable cells was also evident however these did not attach during the expansion of cell numbers by monolayer culture. Morphometric image analysis of bead sections immunolocalised for type I and II collagen and aggrecan demonstrated a steady increase of these components in the alginate beads over 2-4 weeks in culture (Figure 4b,c). Type I collagen was not expressed by rounded bead sections immunolocalised for type I and II collagen but did not express type I collagen (Figure 4e) however it was produced by a few (<5%) cells of a flattened morphology at the bead surface after 4 weeks of bead culture. These expressed low levels of type I collagen (Figure 4e) but did not express type II collagen (Figure 4f). Comparative immunolocalisation of type II collagen (Figure 4b), aggrecan (Figure 4c) and KS (Figure 4d) produced by the central rounded loose body cells in 28 days of alginate bead culture clearly established their pedigree as a chondrogenic cell type.

Discussion

The present study arose from a series of failed attempts to isolate articular chondrocytes from the articular remnants of discarded surgical material from total knee arthroplasty patients. This was not an unexpected finding given that these specimens had little residual cartilage, displayed severe eburnation, loss of >50% of the menisci and marginal osteophytic features of advanced knee-joint degeneration. The chondrocytes that were isolated typically were 50-60% viable, had poor replicative capability, and were obtained in insufficient numbers to warrant further investigation. Incidental observations on a number of these knee replacement tissues drew our attention to small glistening loose bodies present in a significant number of cases (12/18). On closer inspection histologically we were surprised to see that the loose bodies were highly cellular and contained abundant proteoglycan, the same could not be said of the residual articular cartilage of these joints. A necrotic core was a common finding particularly in the loose bodies ≥4 mm in size however this was to be expected given the advanced degenerative features of these knee specimens. We subsequently developed a protocol to isolate the loose body chondrocytes at ≥95% viability.

The therapeutic use of autologous chondrocytes in isolation or in combination with mesenchymal stem cells in the development of cartilage repair strategies are of considerable interest in repair medicine. The present study describes a simple convenient procedure for the isolation of these cells from loose bod-
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Brief Report

ies in the knee joint and could be of further application. We initially examined the cells isolated by flow cytometry, front and side scatter data demonstrated a single cell population with only a small proportion of dead or non-viable cells. Loose body cell numbers were initially expanded in monolayer culture which eliminated these dead cells and demonstrated a homogeneous cell population of cells with a typical cobblestone morphology and high replicative capability and viability. Furthermore, our laboratory has formerly used this culture system with articular chondrocytes.11,13,14,18-20 The microcarrier cell culture system was first introduced in 1967 by van Wezel.21 These spheres were typically 125-250 μm in size and made from DEAE-dextran, bioglass, polystyrene, acrylamide, collagen or alginate and were available commercially as dextran beads (Cytodex, GE Healthcare), alginate (GEM, Global Cell Solutions), collagen (Cultispher, Percell) and polystyrene (Solohill Engineering). The surface chemistries of these microspheres were conducive to cell attachment and they were robust enough for use in spinner cultures. Cancer cells do not divide in this culture system but produce cartilage specific ECM components. The lack of type I collagen synthesis was further evidence of the chondrocytic pedigree of the isolated cell population. Furthermore, morphometric image analysis of the immunolocalised bead sections over a 2-8 week culture period demonstrated a steady increase in chondrocyte ECM products plateauing at 4 weeks of culture. Our laboratory has formerly used this culture system with intervertebral disc cells, meniscal cells and articular chondrocytes.11,13,14,18-20

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Figure 3. Assessment of the polydispersity of the loose body cell preparation isolated in this study and morphometric image analysis of staining intensities of individual beads stained with antibodies to aggrecan, type I, II collagen in alginate bead culture. a) Flow cytometric analysis of the loose body cell preparation isolated by front and side scatter characteristics; the gated area labelled with an asterisk designates dead or non-viable cells. b) Immunolocalised Collagen I, II and aggrecan in bead sections from 2, 3 and 4 weeks of culture (4 beads per time point) were subjected to morphometric image analysis to quantify the staining using Adobe Photoshop software. Integrated density of the stained pixels were measured, data shown is x 106. Immunolocalisation of aggrecan (c,F) and type II collagen (d,g) in sections of beads from 2 and 3 weeks of alginate bead culture.
defined rounded chondrocytic morphologies. A re-evaluation of the microsphere culture system has occurred in the last few years where cancer cells are encapsulated in an environment permissive to cell-cell cross-talk. Various matrix components can also be introduced into the bead in an effort to develop a more appropriate cell culture micro-environment similar to that found in vivo. This culture system has been used to culture macrophages and fibroblasts in breast cancer, invasive hepatocellular cells, and epithelial-stromal cells in prostate cancer. Porous chitosan-alginate microspheres have also been developed to examine prostate cancer and glioma. Hepatocarcinoma spheroids have also been prepared using gelatin microspheres, and controlled release rhBMP2 in 3D printed porous hydroxyapatite, injectable nanofibrous microspheres, and arginine-chitosan BMP-2 nanoparticle cell delivery vehicles for bone repair have also been developed.

These promising initial findings with the loose body chondrocytes warrants further studies to determine the gene expression profiles of the loose body chondrocytes compared to articular and growth plate chondrocytes. Human chondrocytes are difficult to source and it is only relatively recently that knee chondrocytes have become available commercially with most suppliers previously using hip cartilage as a tissue source. Pascual-Garrido et al. found the viability of loose bodies from paediatric patients were 88% vs 92% for healthy articular cartilage. Others have also suggested that the loose body cells represent a valuable resource for autologous cell transplantation. Thus, the loose bodies examined in the present study should be considered a valuable cell resource rather than as a surgical discard.

References

10. Melrose J, Smith S, Cake M, Read R, Whitelock J. Comparative spatial and temporal localisation of perlecan, aggrecan and type I, II and IV collagen in the ovine...