THE IDENTIFICATION AND CHARACTERISATION OF ARTICULAR CARTILAGE PROGENITOR CELLS

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Introduction: There are two major problems which afflict current strategies in cartilage repair. One problem is tissue integration between host and reparative tissue. The second problem is the generation of a repair tissue with the structural characteristics of articular cartilage. Using the marsupial *Monodelphis domestica* as a model system, it has been shown that articular cartilage grows by apposition from the articular surface towards the subchondral bone and that this growth is driven by the proliferation of surface zone cells (1, 2), Additionally, a population of cells with an increased cell cycle time was identified within the surface zone; a property typical of many progenitor cell populations (2). The aim of our research is to identify and characterise a chondroprogenitor population from articular cartilage to enable the rapid culture of undifferentiated chondrocytes in vitro for future clinical use. Here we describe the isolation and partial characterisation of a cell population from the articular surface which exhibits differential adhesion to fibronectin, differential integrin expression and the ability to form large numbers of colonies from an initialy small seeding density; properties that are common to known progenitor cell populations of other tissues. Additionally we report on the presence of the cell surface signalling molecule Notch 1 (N1) in a subpopulation of surface zone chondrocytes and that this N1-expressing subpopulation has an enhanced ability to form large numbers of colonies from an initially low seeding density.

Materials and Methods: *Tissue culture and differential adhesion assay:* Cartilage slices were isolated from the surface (SZ), middle (MZ) and deep (DZ) zones of 7 day old bovine metacarpal-phalangeal joints by fine dissection. Slices were then incubated in pronase (0.1% in DMEM/5%FCS) for 3 hours at 37^{o}C followed by collagenase (0.04% in DMEM/5%FCS) for 16 hours at 37^{o}C. Chondrocytes were counted and seeded onto fibronectin (10µg ml^{-1})-coated or PBS/1% BSA-coated 35 mm dishes at 4,000 cells ml^{-1} in serum free DMEM (DMEM-) for 20 minutes. After 20 minutes, media and non-adherent cells were removed and placed in similarly treated dishes for a further 40 minutes before this media and nonadherent cells were placed in a third dish. After removal of media at 20 and 40 minutes, fresh DMEM- was added to the remaining cells which were cultured for up to 10 days. In all experiments 6 fibronectin and 6 uncoated dishes were used for each zone of cartilage. Fibronectin was used as a ligand in the experiments since it is known to be differentially expressed at the articular surface during mammalian development (3). Within three hours of plating, chondrocyte adhesion was assayed by counting the total number of cells per dish using phase contrast microscopy and expressed as a percentage of the initial seeding density. Additionally, colonies of chondrocytes consisting of 32 or more cells were counted at 0, 3, 6 and 10 days after differential adhesion (n = 6 experiments). Colony forming efficiency (CFE) was calculated by dividing the number of colonies by the initial number of adherent cells. In some experiments (n = 3) the number of cells per colony were counted to determine the average number of cells per colony. Results were analysed using the Students *t* test. *Flow cytometry*: Four hours after differential adhesion, chondrocytes were removed from dishes non-enzymatically and 2 x 10^{5} cells were incubated for 3 hours with antibodies to α5 (AB1928) and β1 (MAB1951) integrin subunits and anti-N1 (SC 6014) at room temperature. Cells were centrifuged at 3,000rpm, supernatants removed and cells washed three times in PBS with centrifugation between each wash. Cells were then incubated with relevant FITC conjugated secondary antibodies for 1 hour at room temperature and washed three times in PBS as described above. Finally, labelled cells were resuspended in 500µl PBS and subjected to flow cytometry. *Notch 1 Immunolabelling and Immunomagnetic Isolation:* Frozen sections of 7 day bovine full depth articular cartilage were immunolabelled with anti-N1 antibody and localised with the appropriate secondary FITC conjugated secondary antibody. Chondrocytes were isolated by sequential pronase/collagenase digestion from surface middle and deep zone articular cartilage and incubated with M450 tosyl-activated Dynal beads conjugated to goat anti-human N1 antibody for 4 hours at 4^{o}C. N1 selected cells were counted and 4,000 cells ml^{-1} subjected to differential adhesion to fibronectin for 20 minutes. Initial adhesion and CFE were assessed as described above.

In all experiments, results were analysed using Students *t* test.

Results: Initial adhesion ranged between 3.5% and 14.5% of the original cell number. Significant differences in adhesion were evident between surface zone chondrocytes seeded on fibronectin for 20 minutes (9.05% +/- 0.44) and those seeded on PBS coated dishes for 20 minutes (3.83% +/- 0.27; $p < 0.001$) and also with those seeded on fibronectin for 40 minutes (4.89% +/- 0.43; $p < 0.001$). Middle zone chondrocytes were significantly more adherent at 20 minutes (14.53% +/- 0.86) than at 40 minutes (10.58% +/- 0.51) when seeded onto fibronectin-coated dishes ($p < 0.01$). Additionally, middle zone chondrocytes were more adhesive to fibronectin at both time points compared with PBS-coated dishes ($p < 0.001$). No differences in adhesion were observed between deep zone chondrocytes regardless of substrate or time point ($p > 0.05$ in all cases).

At days 0 and 3, no colonies containing 32 or more cells were present in any sample. At 6 and 10 days, the CFE of surface zone chondrocytes initially cultured on fibronectin for 20 minutes was greater than that of the other samples ($p < 0.01$ at 6 days, $p < 0.001$ at 10 days). In addition, the CFE of surface zone cells initially cultured for 20 minutes on fibronectin was greater at 10 days compared with that at 6 days ($p < 0.05$). No change in CFE was evident between 6 and 10 days for any other sample ($p > 0.05$ in all cases). The average number of cells per colony was greater in surface zone cells initially grown on fibronectin for 20 minutes at both 6 ($p < 0.05$) and 10 ($p < 0.01$) days compared with all other samples. FACS analysis showed elevated levels of both α5 and β1 integrin subunits in surface zone cells compared with middle and deep zone cells ($p > 0.05$).

N1 immunolabelling revealed occasional N1 positive cells within uppermost 2-3 cell layers of the articular cartilage. When surface zone cells were isolated and analysed for N1 using FACS over 84% of the surface zone population were N1 positive and this result was reflected in the cell counts obtained after N1 selection. Adhesion assays performed using N1 selected chondrocytes revealed that the N1 positive cells were more adherent than either negative cells or unselecetd cells ($p > 0.05$) and that the CFE of N1 selected cells was increased 4 fold relative to negative cells and unselected cells ($p > 0.001$).

Discussion: The ability of a population of cells to form large numbers of chondrocyte colonies from a low seeding density, differences in α5β1 integrin subunit expression and differentail N1 expression when taken together with previous results demonstrating the prolonged cell cycle time at the articular surface (2), strongly suggest that a subpopulation of progenitor chondrocytes resides in the articular surface. Additionally, the prolonged adhesiveness of mid zone cells, their restricted ability to form large numbers of colonies and their relatively short cell cycle (2) strongly indicates the presence of transit amplifying cells within this zone. Furthermore, the use of N1 selection increases the CFE of surface zone cells seeded on fibronectin fourfoldrelative to unselected cells suggesting that N1 will be a useful marker in the further purification of chondroprogenitor cells. The eventual isolation and purification of such a progenitor population will prove to be vital in advancing staetegies for cartilage repair.

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