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Clonal chondroprogenitors maintain telomerase activity and *Sox9* expression during extended monolayer culture and retain chondrogenic potential

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Summary

Objective: Articular cartilage contains mesenchymally derived chondroprogenitor cells that have the potential to be used for stem cell therapy. The aim of this study was to characterise the growth kinetics and properties of *in vitro* expanded cloned chondroprogenitors and determine if critical determinants of the progenitor phenotype were maintained or lost in culture.

Methods: Chondroprogenitors were isolated from immature bovine metacarpalphalangeal joints by differential adhesion to fibronectin. Cloned colonies were expanded *in vitro* up to 50 population doublings (PD). Growth characteristics were assessed by cell counts, analysis of telomere length, telomerase activity, expression of senescence-associated β-galactosidase activity and real-time quantitative polymerase chain reaction to analyse the gene expression patterns of *sox9* and Notch-1 in chondroprogenitors.

Results: Cloned chondroprogenitors exhibited exponential growth for the first 20 PD, then slower linear growth with evidence of replicative senescence at later passages. Mean telomere lengths of exponentially growing chondroprogenitors were significantly longer than dedifferentiated chondrocytes that had undergone a similar number of PD ($P < 0.05$). Chondroprogenitors also had 2.6-fold greater telomerase activity. Chondroprogenitors maintained similar *sox9* and lower Notch-1 mRNA levels compared to non-clonal dedifferentiated chondrocytes. Chondroprogenitors were induced to differentiate into cartilage in 3D pellet cultures, immunological investigation of *sox9*, Notch-1, aggrecan and proliferating cell nuclear antigen (PCNA) expression showed evidence of co-ordinated growth and differentiation within the cartilage pellet.

Conclusion: Clonal chondroprogenitors from immature articular cartilage provide a useful tool to understand progenitor cell biology from the perspective of cartilage repair. Comparisons with more mature progenitor populations may lead to greater understanding in optimising repair strategies.

Key words: Chondroprogenitor, *sox9*, Notch-1, Telomerase, Telomere.

Introduction

The use of stem/progenitor cells whose progeny have the capacity to form good quality cartilage matrix has been targeted as a therapeutic goal for the treatment of large defects of articular cartilage that may result from serious trauma or widespread osteoarthritic lesions. Currently, smaller more localised lesions have been treated with expanded mature chondrocytes harvested autologously and expanded in monolayer culture[1–4]. A major limiting factor of this technique is that the size of defect to be treated is dependent on the amount of cells that can be generated from the harvested cartilage taken from the joint periphery. The reason for this limitation is that during expansion in monolayer culture, chondrocytes undergo progressive non-chondrogenic phenotypic modulation as a function of mitotic index and, in human chondrocytes, are unable to re-express the cartilage phenotype after 5–7 population doublings (PD), even under permissive pellet culture conditions where the characteristic rounded morphology of the chondrocytes is re-established[5–8]. Although the number of cell cycles can be extended somewhat by addition of fibroblast growth factor-2, the generation of large cell numbers is problematic. Consequently, there is a clinical need for increasing cell yield whilst maintaining chondrogenic potential in order to address the treatment of larger cartilage defects.

We have previously reported on the isolation and partial characterisation of a progenitor cell population that resides in the surface layer of neonatal bovine articular cartilage[9]. Here, we report on the long-term clonal expansion of the progenitor cells in monolayer culture and assess their chondrogenic potential as a function of their ability to elaborate a cartilage matrix in pellet cultures, maintenance of *sox9* expression, analysis of telomere length and telomerase activity.

Materials and methods

ISOLATION AND CULTIVATION OF CHONDROPROGENITOR CLONES

Chondroprogenitor cells were isolated on the basis of differential adhesion to fibronectin as previously described in Dowthwaite *et al.*[9] Firstly, surface-zone chondrocytes were isolated by surgical dissection from the metacarpalphalangeal (MCP) joints of 7-day-old juvenile bovine steers and subjected to sequential

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pronase, collagenase enzymatic digestion. Isolated chondrocytes were subjected to differential adhesion on fibronectin-coated 60 mm dishes for 20 min in 4 ml 1:1 Dulbecco's modified eagles medium (DMEM)/F12 plus 10% foetal calf serum (Invitrogen, UK) at a concentration of 700 cells ml^{-1}. After 20 min, media and non-adherent cells were removed and replaced with standard growth media. Cultured cells were maintained in a humidified 37°C, 5% CO_2 incubator for 6 days. Colonies of >32 cells (chondroprogenitor clones) were isolated using cloning rings and replated in 35 mm culture dishes and further expanded. Enriched (polyclonal) populations of chondroprogenitors were culture expanded from the 60 mm dishes used in the initial adhesions. Full-depth and superficial zone cells were enzymatically isolated using surgical dissection from the MCP joints. Cells were passaged in T25 culture dishes and on reaching confluence were trypsinised and replated at a ratio of 1:10. Cell counts were recorded at the end of every passage and the number of PD calculated using the following equation: $PD = \log_{10}(N/N_0) \times 3.33$, where N_0 is the initial number of cells seeded and N is the number of cells at the end of the passage.

CHONDROPROGENITOR PELLET CULTURE

Chondroprogenitor clones (average 30 PD, $n = 6$) were pelleted at a concentration of 1×10^6 cells per 1.5 ml Eppendorf centrifuge tube and grown in chondrogenic medium; DMEM/F12, 1% insulin–transferrin–selenium supplement (Sigma), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 0.1% gentamicin, 0.1 μM dexamethasone and 10 ng transforming growth factor β1 (TGFβ1) (PeproTech EC Ltd., London, UK). The chondrogenic medium was replaced every other day and pellet cultures maintained for a total of 21 days.

TELOMERE-LENGTH ASSAY

Telomere lengths of samples were detected using the TeloTAGGG telomeric length assay kit (Roche Diagnostics, Sussex, UK). The mean telomere length of samples was obtained by scanning the exposed X-ray film with a densitometer within the linear range of the film. Each lane was split into 30 equally spaced squares (i) and the optical density (OD) within each square was obtained as well as the molecular weight at the mid point of the corresponding square. The telomere length was then calculated using the expression, mean telomere length $= \sum(OD_i)/\sum(OD_i/L_i)$.

REAL-TIME QUANTITATIVE TELOMERE REPEAT AMPLIFICATION PROCEDURE (RTQ-TRAP)

Comparative quantitative analysis of telomerase activity in samples was performed using a previously validated RTQ-TRAP methodology[10]. Cells were lysed at a concentration of 1×10^6 per 200 μl of lysis buffer; 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 10% glycerol, 0.5% 3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid buffer, 1 mM phenylmethanesulphonylfluoride, 5 mM β-mercaptoethanol and 200U RNAsin. Each reaction comprised 1× SYBR Green Mastermix (Eurogentec Ltd., Hampshire, UK), 10 mM EGTA, 0.2 μg T4 gene protein, 0.3 μM primers TS (5′-AAT CCG TCG AGC AGA GTT-3′) and ACX (5′-GCG CGG [CTT ACC]$_3$ CTA ACC-3′) and 25×10^3 cells in a final volume of 25 μl. The reaction mixture was incubated for 25°C for 20 min to allow the telomerase to extend the TS primer, heated to 95°C for 10 min to activate Taq Polymerase, followed by 40 cycles at 95°C for 20 s, 50°C for 30 s and 72°C for 90 s. Telomerase activity in cell samples was calculated based on the threshold cycle (C_T). All samples were run in triplicate and lysis buffer was used as a negative control. Immortal human leukaemia cell line HL60 was used as a cell-line positive for telomerase activity. HL60 cell extracts were serially diluted and assayed by RT-TRAP to generate a standard curve that was used to derive telomerase activity units based on the number of input cell equivalents.

DETECTION OF SENESCENT-ASSOCIATED β-GALACTOSIDASE (SA β-GAl) ACTIVITY

Senescence was detected by observation of endogenous β-galactosidase (β-Gal) activity at pH 6.0. Cells were fixed in 2% formaldehyde/glutaraldehyde for 4 min, and then incubated (18 h, 37°C) in reaction mix (40 mM citric acid/phosphate-buffered salts (PBS) pH 6.0, 1 mg/ml X-Gal, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$)[11]. Senescent cells stained blue. Results were expressed as the percentage of positively labelled cells. A total of four random fields of view from each dish were examined.

REAL-TIME POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION AND QUANTITATIVE ANALYSIS

Quantitative polymerase chain reaction (qPCR) using the fluorescent dye SYBR Green (Eurogentec, Belgium) was used to determine the absolute expression levels of collagen type II, aggrecan, *sox9*, *HES-1* and Notch-1 between different cell populations. The PCR products from reactions containing the following primer pairs; *sox9* (forward: AAC GCC GAG CTC AGC AAG, reverse: ACG AAC GGC CGC TTC TC), Notch-1 (forward: TGA GCA CGC GGG CAA GTG CAT, reverse: TGC AGA CAC TGG CAC TCG A), Hairy Enhancer of Split-1 (HES-1; forward: GCC TCC ACC TAA ACG ACT CA, reverse: TTG ATC CCC CTC GCT CTT TTA) and 18S rRNA primers, were cloned and sequenced to confirm their identities. The sequences of collagen type II, 18S and aggrecan primers have been previously published[12]. Real-time PCR reactions were carried out in 25 μl volumes in a 96-well plate (Applied Biosystems™) containing 1× buffer (10×), 3.5 mM $MgCl_2$, 200 μM dNTPs, 0.3 μM of sense and antisense primers, 0.025 U/μl enzyme and 1:66000 SYBR GreenI®. All reactions were made using qPCR™ Core Kit for SYBR Green I® (Eurogentec). At the end of each reaction, the cycle threshold (C_T) was manually setup at the level that reflected the best kinetic PCR parameters, and melting curves were acquired and analysed. Absolute values for the gene of interest were calculated from standard curves generated using serially diluted plasmid cloned and sequence verified template (ng DNA) and were normalised to the housekeeping gene 18S rRNA. Relative expression levels for monitoring *sox9* levels between chondroprogenitor clones at increasing passages were determined using the $\Delta\Delta C_T$ method.

ANTIBODY LABELLING OF CHONDROPROGENITOR PELLETS

Wax embedded tissue blocks of 4% paraformaldehyde fixed chondroprogenitor pellets were cut to generate 8 μm sections, mounted onto coated slides and processed for immunodetection of proteins. Primary antibodies, monoclonal anti-rat Notch-1 (bTan20, Developmental Studies Hybridoma Bank, Iowa, USA), monoclonal anti-bovine aggrecan (2B6), monoclonal anti-mouse proliferating nuclear antigen (PCNA; PC10, Sigma) and rabbit anti-*sox9* (ab3697, Abcam, UK) were diluted in tris-buffered salts (TBS) at a concentration of 5 μg ml^{-1} and sections probed for 12 h at 4°C. Sections were probed with either anti-goat peroxidase-conjugated or fluorescein isothiocyanate (FITC)-conjugated anti-rabbit and anti-mouse secondary antibodies (Sigma). Peroxidase-conjugated antibodies were detected on sections using colour substrate (Vector SG, Vectorlabs) and then counterstained for 30 s in 0.1% Safranin-O. Fluorescently labelled sections were mounted in Vectashield (VectorLabs) containing 4′,6-diamidino-2-phenylindole (DAPI) as a counterstain for cell nuclei and viewed using an Olympus BX61 fluorescence microscope. We used goat, mouse and rabbit serum at the same concentrations as the primary antibodies to control non-specific labelling in sections.

STATISTICAL ANALYSIS

The data are reported as mean ± standard deviation of the indicated number of experimental values. All datasets were assessed for normal population distribution using the Shapiro–Wilk test, and homogeneity of variance using Levene's test. One-way analysis of variance (ANOVA) was used to compare means of datasets that met all the assumptions of this test, followed by *post hoc* pairwise comparisons using the method of Bonferroni. Where assumptions for ANOVA were not met, non-parametric Kruskal–Wallis ANOVA was employed using Mann–Whitney U tests for subsequent pairwise comparisons. In all cases, $P < 0.05$ was considered statistically significant. All experiments were repeated at least twice.

Results

Clonally derived chondrocyte progenitor cells were isolated from juvenile bovine chondrocytes using differential adhesion to fibronectin and expanded in monolayer culture. Clonal chondroprogenitor morphology after increasing PD was recorded, Fig. 1(A). Early passage chondroprogenitors were small and polygonal, whereas after extended monolayer cultivation (45 PD) progenitor cell bodies had enlarged significantly and were multipolar, Fig. 1(A).

The growth kinetics of chondroprogenitor clones ($n = 8$) was investigated beginning from their isolation as colonies of greater than 33 cells through to 17 passages corresponding to an average of 47 ± 4.4 PD, Fig. 1(B). Passage-1 cultures reached confluence within 2 weeks undergoing on average 20 ± 2.3 PD, displaying a doubling time of less than 24 h reflecting the time progenitors were under the influence of rapid growth. During subsequent passaging, progenitor clone proliferation rates slowed to 3–4 days per PD, which with further cultivation declined to 1 PD per week. At