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Molecular and phenotypic modulations of primary and immortalized canine chondrocytes in different culture systems

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ABSTRACT

This study was conducted to determine physiological and functional features of primary and immortalized canine chondrocytes. Chondrocytes were immortalized by introducing the catalytic component of human telomerase namely human telomerase reverse transcriptase (hTERT). Primary chondrocytes lost their characteristic phenotype and growth properties whereas the immortalized cells remained polygonal with rapid growth rate. The expression of chondrocyte-specific markers decreased many-fold whereas that of chondrocyte-non-specific gene increased in primary chondrocytes. The immortalized cells did not express chondrocyte-specific genes in monolayers. Both primary and immortalized cells were encapsulated in alginate microspheres to construct three-dimensional (3D) culture system. As the primary chondrocytes, also the telomerase-transfected cells adopted a chondrocyte-specific gene expression pattern in alginate culture. Thus, the expression of telomerase represents possibility to expand chondrocytes without limitation while maintaining the chondrocyte-specific phenotype in 3D cultures. Use of such cells provides a standardized tool for testing different tissue engineering applications in canine model.

1. Introduction

Articular chondrocytes are specialized cells of mesenchymal origin found exclusively in cartilage. The ease of recovery of the integral chondrocytes and their adaptability to cell culture makes these an attractive source of tissue engineering applications. However, on prolonged culturing in monolayers (2D culture), the chondrocytes de-differentiate into fibroblastic type cells (flattened and elongated morphology) with a reduced expression of the characteristic chondrocyte phenotype (von der Mark et al., 1977). De-differentiation is a reversible process characterized by the cessation of collagen type II mRNA and protein synthesis and the induction of collagen type I mRNA and protein synthesis. Typically, the de-differentiated chondrocytes show increased collagen type I levels (Marlovits et al., 2004a) and decreased ability to express certain extracellular macromolecules, such as proteoglycans (van Osch et al., 1998), collagen type II, aggrecan (Marlovits et al., 2004b), cartilage acidic protein (CRTAC)-1 (Steck et al., 2001), cartilage oligomeric matrix protein (COMP) (Zaucke et al., 2001) and SRY (sex determining region Y) Box-9 (SOX-9) (Tew and Hardingham, 2006). To circumvent this problem, an alternative three-dimensional (3D) cell culture was introduced that reverses this de-differentiation phenomenon (Watt and Dudhia, 1988; Guo et al., 1989; Bonaventure et al., 1994; Binette et al., 1998; Madry et al., 2003). Of all different types, alginate beads offer a convenient matrix to form 3D cultures and also to recover the cells by solubilization. The other commonly used method is based on agarose which was reported to cause RNA extraction problems for PCR based assays (Kuroki et al., 2005). Alginate beads have also been successfully used for *in vivo* studies in rabbits (Madry et al., 2003). Moreover, the authors in the same report observed that alginate could avoid antigenicity when allogenic chondrocytes are transplanted into rabbits. The dog model for the study of arthritis offers benefits, as damage to cartilage and associated arthritis is a common phenomenon. Additionally, dog being a large animal offers a promising model to compare the prognosis of tissue engineering for use in humans. Previously canine intervertebral disk chondrocytes were used in alginate microspheres (Masuda et al., 2002) and recently canine articular chondrocytes (CAC) have been used in this 3D culture system (Nicholson et al., 2007).

The other aspect for the present study is the generation of a stable CAC cell line that offers infinite proliferation capacity and a stable phenotype. Until now, several immortalized chondrocyte lines of animal or human origin have been successfully developed through viral transfection with selected single or multiple

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oncogenes (Goldring et al., 1994). Since the use of such oncogenic cell lines in tissue engineering is not warranted for safety reasons, our present work focuses on safer means of using human telomerase reverse transcriptase (hTERT) in order to develop a stable cell line that could be used both *in vitro* and probably also for *in vivo* experiments. The hTERT is a human telomerase catalytic subunit that adds telomeric repeats to the ends of human chromosomes and has been shown to induce cell immortalization. Available evidence suggests that the regulation of telomerase activity primarily depends on transcriptional control of hTERT (Colgin et al., 2000). Although the effects of expression of exogenous telomerase have been investigated in a number of human somatic cell types and chondrocytes, until very recently there were no studies on this issue. While our study was emerging, a first report by Nicholson et al. (2007) showed that hTERT can extend the lifespan of canine chondrocytes *in vitro* without inducing neoplastic transformation.

Our present work adds to the body of knowledge by thorough analysis of chondrocyte marker genes from both primary to hTERT stabilized CAC in both a 2D monolayer and 3D alginate culture system. Additionally, growth kinetics on primary chondrocytes were also studied which supports the need for a stable chondrocyte cell line. Chondrocyte cell lines as the telomerase transfected one described here adds to the list of *in vitro* tools for future research in arthritis and inflammation.

2. Materials and methods

2.1. Cartilage specimens collection

Articular cartilage was collected from macroscopically normal stifle joints exclusively from femoral condyles of five canine cadavers (age range: 4 months to 7 years) 24 h after euthanasia. Samples were prepared for cell isolation under a laminar flow hood. Chondrocytes from one of the donors were used for characterization to immortalization based on initial growth characteristics.

2.2. Isolation of chondrocytes

Chondrocytes were isolated according to previously described protocol (Kaps et al., 2004) with slight modifications. Briefly, cartilage was sliced by careful dissection and precautions were taken to avoid inclusion of the subchondral bone. Samples of cartilage were collected in Dulbecco's Modified Eagle Medium (DMEM; Pan Biotech, Germany) enriched with 10% heat inactivated fetal calf serum (FCS; BioWhittaker Germany), 100 mg/ml streptomycin and 100 U/ml penicillin ($DMEM^{++}$). The cartilage was washed thrice with HANKS' salt solution, once with 70% ethanol and twice with $DMEM^{++}$. Washed samples were transferred into Petri-dishes, diced into pieces of 1–3 mm^2, medium was aspirated and cartilage slices were placed in spinner flasks. A cocktail of enzymes consisting of 1 U/ml Collagenase P (Roche Diagnostics, Germany), 330 U/ml Collagenase CLS II (Biochrom, Germany) and 30 U/ml Hyaluronidase (Roche Diagnostics, Germany) was added in the flasks. The spinner flasks were placed in an orbital shaker at 37 °C with 5% CO_2 and 95% humidity. After 16–18 h the digest was filtered through nylon mesh (pore size 100 μm) centrifuged at 400*g* for 20 min and the cell pellet was washed thrice in HANKS' salt solution. The cells were counted using a haemocytometer, and viability was determined by trypan blue exclusion test.

2.3. Chondrocyte culture in monolayers

Freshly isolated chondrocytes were seeded in tissue culture dishes or flasks as the primary culture (P0) at a density of 20,000 cells/cm^2 in $DMEM^{++}$. When the P0 reached confluence, it was trypsinized with 0.05% trypsin–EDTA (BioWhittaker Germany). The harvested cultured chondrocytes were washed thrice in PBS^{-1} (without calcium and magnesium). The resulting cell pellet was resuspended in 10 ml of $DMEM^{++}$ for total cell count and cell viability. Cultured chondrocytes were then sub-cultured (passage 1; P1) into new tissue culture dishes under previously described culture conditions. Once confluent, it was sub-cultured for another five passages (P2 to P7). Total cell number and cell viability were recorded at every passage.

2.4. Chondrocytes in alginate culture

CAC culture was continued until approximately 80% confluency at which point the cells were freed by trypsinization, then the number and viability were recorded. Cells were centrifuged at 200*g* for 5 min and the resuspended pellet was encapsulated in alginate microspheres essentially as previously described (Madry et al., 2003). Chondrocytes (passage 3) were suspended in 1.2% alginate in 0.15 M NaCl solution at 5×10^5 cells/ml. The cell suspension was extruded into a 102 mM $CaCl_2$ solution under constant shaking and the chondrocytes–alginate composite was allowed to polymerize for 10 min. The resulting implants were then washed twice in 0.15 M NaCl followed by two consecutive washes in basal medium and placed in growth medium that was changed three times per week or as and when required for defined time periods as described under results. Encapsulated chondrocytes were released from alginate beads as mentioned below: Single spheres were solubilized by individual incubation in 100 μl of 55 mM sodium citrate and 90 mM NaCl (pH 6.8) solution for 20 min at RT. The released chondrocytes were pelleted for total RNA isolation.

2.5. Growth characteristics and phenotype of chondrocytes

CAC were cultured until the 7th passage in monolayer and the growth rate during cultivation was estimated using the growth constant $k = (\mathrm{In}N_2 - \mathrm{In}N_1)/t$ in which the initial number of cells ($N_1 = 1 \times 10^6$ cells), the number of cells in confluent cultures (N_2), and the number of days required for reaching confluence (t) were used to calculate the value of k. Cell morphology was first analyzed visually by light microscopy of confluent cultures. Samples of cells obtained after the isolation and trypsinization were then analyzed by flow cytometry (Beckman Coulter® Flow Cytometer, Canada) for investigation of the forward light scatter (FSC) and the orthogonal or side light scatter (SSC) profile of harvested cells as described earlier (Fröhlich et al., 2007).

2.6. Histological and immunohistochemical analysis of cartilage tissue

Cryostat sections (3–5 μm thick) were cut from cartilage tissue and frozen at −70 °C. Sections, on super-frost slides were fixed for 5 min in ice-cold acetone and then stored at −20 °C until required for alcian blue staining which reacts with proteoglycans, a component of cartilage matrix, or for immunostaining to detect collagens in the tissue.

The cryosections from the central part of the tissue were mounted on aminoalkylsilane-coated slides. Cartilage matrix proteoglycans were stained with alcian blue 8GX (Roth, Karlsruhe, Germany) at pH 2.5 and counterstained with nuclear fast red (DAKO, Hamburg, Germany) according to the method of Romeis and Boeck (1989).

For the detection of collagen type I or collagen type II, cryosections were incubated with goat anti-collagen type I polyclonal (dilution, 1:1000) or rabbit anti-bovine collagen type II polyclonal antibody (dilution 1:200). The slides were then washed three times with PBS^{-} and incubated for 30 min at 37 °C with LSAB/LINK,