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Low calcium levels in serum-free media maintain chondrocyte phenotype in monolayer culture and reduce chondrocyte aggregation in suspension culture

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Summary

Objective: Extracellular calcium influences chondrocyte differentiation and synthesis of extracellular matrix. Previously, calcium concentrations ranging from 0.1 mM to 2 mM have been used *in vitro* and these studies indicated that low calcium concentrations were generally favorable for chondrocyte culture. Our objective was to extend these findings to yet lower calcium concentrations and to comprehensively examine effects on morphology and phenotype in two culture systems.

Methods: Serum-free media containing 1 mM, 50 μM or 15 μM of calcium and a serum-containing medium were used to culture chondrocytes in suspension and in monolayer, at high and low inoculation density.

Results: In monolayer, at low and high density, removing serum and decreasing calcium concentration decreased cell spreading and lowered collagen type I expression whereas collagen type II expression remained stable. In suspension, cells aggregated for all media tested; however, aggregates were smaller and looser in the absence of serum.

Conclusion: The serum-free 50 μM and 1 mM calcium media provide good alternatives to classical media for monolayer culture since both growth and chondrocyte phenotype were maintained. In suspension culture, the serum-free 1 mM calcium medium also possesses the beneficial properties of limiting aggregate size while maintaining growth and phenotype.

Key words: Chondrocyte, Calcium, Cell aggregation, Chondrocyte phenotype, Suspension culture.

Introduction

In monolayer culture, primary articular chondrocytes dedifferentiate to a fibroblastic phenotype expressing collagen type I in preference to collagen type II[1,2]. In contrast, culturing chondrocytes in three dimensional (3D) gels effectively maintains the chondrocyte phenotype, although at the expense of lowering cell division[3–6]. These latter phenotypically stable systems are, however, spatially inhomogeneous since cells are exposed to different microenvironments depending on their position within these 3D systems. Chondrocytes have also been cultured in suspension[7,8] where chondrocyte phenotype was promoted, however large cell aggregates formed quickly, also leading to the loss of culture homogeneity. Thus, the challenge remains to develop homogeneous culture conditions for primary chondrocytes which allow growth, phenotypic stability and minimize cell aggregation.

Extracellular calcium has strong potentiating effects on cell adhesion and cell aggregation for diverse cell types *in vitro*. In monolayer culture, low calcium content reduces cell spreading in muscle[9], epithelial[10] and endothelial cells[11]. In suspension culture, lowering calcium to 100 μM reduces aggregation of HEK 293 cells[12]. Similarly, calcium removal by chelation with 5 mM ethylene glycol-bis(β-aminoethyl-ether)-N,N,N′,N′-tetraacetic acid (EGTA) inhibited the condensation of limb bud cells, when calcium-dependent aggregation processes were active[13].

Extracellular calcium regulates matrix synthesis of chondrocytic cells. RNA levels for aggrecan and type II collagen in a chondrocyte cell line decreased with increasing initial medium Ca^{2+} concentration (ID_{50} ~2 mM for aggrecan and 4.1 mM for type II collagen)[14]. Changes in calcium concentration in the range of 1–4 mM are also sensed by Ca^{2+} receptors (CaR), that belong to the G protein-coupled receptor superfamily[15]. These CaR are involved in the influence of extracellular calcium on differentiation where high calcium increased expression of osteopontin, osteonectin and osteocalcin in chondrogenic cells[16], and collagen type X in chondrocytes[17]. On the contrary, calcium concentrations below 0.5 mM promoted the production of articular collagens, types II and XI[18]. It has also been shown that using a medium without added calcium (but with 10% serum that contains calcium) has prevented rabbit articular chondrocytes from switching to a collagen type I-producing fibroblastic phenotype when grown in suspension culture[19,20]. Finally, elemental analysis by electron microscopy revealed low extracellular calcium levels (1/4 of the cytosolic concentration that ranges from 0.1 to 10 μM) in the proliferating zone of the cartilage growth plate[21]. Taken together, the above data from the literature strongly suggest that articular chondrocytes exist in a low calcium environment that may be necessary for their physiological function and phenotypic stability.

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Although existing data clearly indicate that calcium has a regulatory role on chondrocytes and that low extracellular calcium may be beneficial to chondrocytes, the lowest calcium concentration reported to date in chondrocyte culture was 0.1 mM. We believed that yet lower extracellular calcium concentrations could bear certain advantages for the culture of primary chondrocytes. We specifically hypothesized that low calcium levels in medium would: (1) promote collagen type II expression over collagen type I expression; (2) promote a chondrocytic (round) cell morphology in monolayer culture and (3) reduce cell aggregation in suspension. Since serum contains about 4 mM of calcium[22], a cell culture medium with calcium concentrations below 0.1 mM required the use of a serum-free medium (SFM). Therefore, an SFM with calcium concentrations of 1 mM, 50 μM, and 15 μM was compared to a standard serum-containing (10%) formulation that had 2 mM calcium. The influence of these different calcium levels, as well as the presence or absence of serum, was investigated using two culture systems: standard monolayer culture as well as suspension culture each at low and high cell-seeding densities.

Materials and methods

CULTURE MEDIA

All reagents were from Sigma–Aldrich Canada, Oakville, Ontario, Canada, unless indicated. Cells were cultured in either a serum-containing medium (SCM) or an SFM. The SCM was chosen to represent a commonly used SCM composed of DMEM low glucose (Life technologies, Burlington, Ontario, Canada) supplemented with 0.4 mM proline, non-essential amino acids 1× (containing 8.9 mg/L alanine, 15 mg/L asparagine, 13.3 mg/L aspartic acid, 14.5 mg/L glutamic acid, 7.5 mg/L glycine, 11.5 mg/L proline and 10.5 mg/L serine), 22 mM sodium bicarbonate, 12.5 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), penicillin/streptomycin 1× (containing 100 U/mL penicillin and 0.1 mg/mL streptomycin)[23]. Medium pH was adjusted to 7.2 and sterile filtered prior to addition of 10% fetal bovine serum (FBS) and 30 μg/mL ascorbate, the latter added fresh just prior to medium change. The SFM was chosen based on commonly used SFM compositions using a 1:1 (v/v) mix of calcium-free HAM's F12 (US Biological, Swampscott, MO, USA) and calcium-free DMEM low glucose (US Biological, Swampscott, MO, USA) supplemented with 0.4 mM proline, 1.5 mM glutamine, 22 mM sodium bicarbonate, non-essential amino acids 1×, 12.5 mM HEPES, penicillin/streptomycin 1×, ITS + (10 μg/mL insulin, 5.5 μg/mL transferrin, 0.05% w/v bovine serum albumin (BSA), 1.7 mM linoleic acid, 0.5 μg/mL sodium selenite), 5×10^{-5} M β-mercaptoethanol and 10^{-8} M dexamethasone[24]. Medium pH was adjusted to 7.2 and sterile filtered. The following recombinant growth factors (from R&D Systems, MN, USA) were added to SFM each at 2 ng/mL: epidermal growth factor (in sterile 0.1% BSA in 10 mM acetic acid), platelet derived growth factor-BB (in sterile 0.1% BSA in 4 mM HCl), fibroblast growth factor-2 (in sterile 0.1% BSA, 1 mM DTT in PBS without Mg^{2+} and Ca^{2+})[24]. Ascorbate, 30 μg/mL, was added fresh just prior to changing SFM. To this SFM was added a supplemental amount of $CaCl_2$ corresponding to 1 mM, 50 μM, 5 μM, 1 μM, or 0 μM. Calcium content in media was then verified by atomic absorption spectrometry with an Analyst 200 from PerkinElmer (Boston, MA, USA) with $CaCO_3$ as a standard (from PerkinElmer). Lanthane chloride (1000 ppm) was added to samples before analysis. This analysis revealed that these media contained in fact, 0.95 mM, 50.25 μM, 18.25 μM, 14.5 μM and 13.75 μM of calcium, respectively. Thus, calcium levels below 10 μM could not be achieved since calcium was present in the additives. Since our results obtained with cultures in 18.25 μM, 14.5 μM and 13.75 μM of calcium were indistinguishable, we only present results obtained with 0.95 mM, 50 μM and 14.5 μM of calcium, indicated by the rounded concentrations of 1 mM, 50 μM and 15 μM.

CELL ISOLATION

Cells were isolated from the femoropatellar groove of a 1–2-month-old calf knee, obtained from a local butcher within 24 h of slaughter. Briefly, cartilage was sequentially digested, first for 90 min by protease Type XIV (Sigma–Aldrich Canada, Oakville, Ontario) 56 U/mL at 37°C in DMEM high glucose supplemented with 22 mM sodium bicarbonate and 1× penicillin/streptomycin, and then for 3 h by collagenase CLS2 (Worthington, Freehold, NJ, USA) 752 U/mL at 37°C in DMEM high glucose supplemented with 22 mM sodium bicarbonate, penicillin/streptomycin and 5% FBS. Released cells were then filtered through a 200 μm mesh (using an autoclaved 200 μm screen mounted on a screen cup, Sigma–Aldrich Canada, Oakville, Ontario), centrifuged (190 *g*, 10 min at 4°C) and filtered again through two serial 20 μm filters using a Swinnex filter holder (Fisher Scientific, Town of Mount-Royal, Quebec, Canada) containing a 20 μm Spectra/Mesh Nylon Macroporous Filter (Spectrum Laboratories, Rancho Dominguez, CA, USA). Cells were then washed three times in SFM without calcium, counted using a hemocytometer and seeded at low or high density in the different culture media and systems described below.

CELL CULTURE

Cells were cultured either in monolayer or in suspension. In monolayer, cells were suspended in 5 mL of culture media and seeded in 60 mm Petri dishes (internal diameter 54 mm, area 23 cm^2). For suspension culture, Petri dishes were previously coated with 2% SeaPlaque low-melting-temperature agarose (Mandel, St. Laurent, Quebec, Canada) in a 1:1 (v/v) mixture of calcium-free HAM's F12 and calcium-free DMEM low glucose or in DMEM low glucose, supplemented with 22 mM sodium bicarbonate, depending on which medium was to be subsequently used. In the SCM, and in 15 μM and 1 mM calcium-containing SFM, cells were seeded at both low density (10^4 cells/cm^2 hence 2.3×10^5 cells/dish) and high density (10^5 cells/cm^2 hence 2.3×10^6 cells/dish) for both monolayer and suspension cultures. To reduce the number of conditions to a manageable level, only low density cultures included the 50 μM calcium-containing SFM condition, in both monolayer and suspension cultures. Half of the media volume was changed every 2 days. In suspension culture, this was achieved by centrifuging cells (190 *g*, 10 min at 4°C) and removing 2.5 mL of the supernatant followed by addition of 2.5 mL of fresh media, that was then resuspended and transferred to the former Petri dish. Each culture condition was done in triplicate and for each result shown, at least one additional preliminary experiment confirmed the general trends of these results, supporting their reproducibility. At day 10, for two of the triplicates, cells were harvested with 1 or 0.5 mL Trizol® (Life technologies, Burlington, Ontario, Canada), depending on cell density,