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Annulus fibrosus cells can induce mineralization: an in vitro study

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Abstract

BACKGROUND CONTEXT: There is still no consensus as to whether the calcification observed in degenerate intervertebral discs (IVDs) is a cause or a consequence of disc degeneration.
PURPOSE: To investigate the mineralization potential of healthy (independent of other associated changes) annulus fibrosus (AF) cells under controlled in vitro conditions.
STUDY DESIGN/SETTING: In vitro study to investigate the mineralization potential of the AF cells.
METHODS: Annulus fibrosus cells, isolated from bovine IVDs, were grown in monolayer. The effect of cell density, culture time, age of cell source, and passage on the percentage of AF cells with alkaline phosphatase activity (ALPa) was evaluated. Gene expression of mineralization-associated markers was determined. Cells were immunostained for Type I, II, and X collagens. To study mineralization potential, AF cells and AF cells that were sorted into two populations, high (top 5%±1%) or low (bottom 5%±1%) ALPa expressors, were grown in the presence of β-glycerophosphate for 2 weeks.
RESULTS: The percentage of AF cells that express ALPa changes with time in culture and seeding density for primary immature and mature cell sources but not for passaged cells. Gene expression of ALP, matrix metallopeptidase-13 (MMP-13), osteopontin, and runt-related transcription factor 2 was upregulated by Day 7. Under mineralization-inducing conditions, high ALPa expressors and unsorted AF cells formed von Kossa–positive nodules, composed of hydroxyapatite as determined by electron diffraction analysis. Low ALPa expressors had significantly fewer von Kossa–positive nodules ($p<.01$) compared with high ALPa expressors. Cells showed colocalization of Type I collagen and ALPa. No Type II collagen was detected suggesting that these were AF cells and not chondrocytes.
CONCLUSIONS: Annulus fibrosus cells have mineralizing capability and form hydroxyapatite crystalline deposits when cultured under appropriate conditions. This system could be used to investigate mineralization mechanisms in the AF during pathological calcification and at the AF-bone interface in disc degeneration.

Keywords: Annulus fibrosus; Mineralization; Alkaline phosphatase; Hydroxyapatite; β-Glycerophosphate

Introduction

The intervertebral disc (IVD) is located between vertebral bodies of the spine and comprises annulus fibrosus (AF), nucleus pulposus (NP), and cartilage end plates [1]. The AF is highly organized and characterized by multiple lamellae, rich in Type I collagen that wrap around the NP

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and insert into the vertebral bodies [2]. The confinement of NP by the AF allows the IVD to resist compressive loads [3,4]. Degeneration of the IVD causes a loss of disc function and can be associated with low back pain [5–7]. The health care and related costs for low back pain exceed $100 billion every year in the United States [8]. Although the pathogenesis of degeneration is yet to be fully elucidated, it does appear to be a complex irreversible process, associated in part with aging. Calcification of the IVD tissues has also been shown to correlate with aging, degeneration, and scoliosis (considered to represent advanced aging) discs [5,7–9].

The process of calcification is very complex and involves the deposition of calcium apatite crystals, with alkaline phosphatase (ALP) playing an active role in initiating this process [10–12]. Alkaline phosphatase hydrolyzes organic phosphate and inorganic pyrophosphate, yielding monophosphate ions, which, in the presence of calcium ions, form hydroxyapatite crystals [13,14]. Cells that have the potential to induce mineralization are known to express a number of genes such as runt-related transcription factor 2 (RunX-2), Type X collagen, osteoprotegerin, and ALP [12,15,16].

Human IVDs of patients with degenerative disc disease and adolescent idiopathic scoliosis have higher ALP activity (ALPa) as well as Ca^{2+} and phosphate concentrations than healthy discs [17,18]. Type X collagen and osteoprotegerin were found to be present only in diseased discs [17,18]. In addition, both osteoprotegerin levels and the number of von Kossa–stained deposits (suggestive of calcification) correlated significantly with Thompson grade, a measure of disc degeneration [17]. There is still no consensus as to whether the calcification observed in degenerate IVDs is a cause or a consequence of disc degeneration [19–21]. Therefore, this study set out to investigate the mineralization potential of a healthy AF tissue independent of other associated changes. The optimal conditions for maximizing AF cell expression of ALPa were identified and then it was determined whether these cells could induce mineralization.

Materials and methods

Cell isolation and culture

The IVDs were aseptically excised under sterile conditions from immature (6–9 months) and mature (18–24 months) bovine caudal spines within 24 hours of death and placed in serum-free Dulbecco's Modified Eagle's Medium (DMEM) containing 1% antibiotics (penicillin G, streptomycin sulfate, and amphotericin B). The outer AF was dissected and subjected to sequential enzymatic digestion in 0.5% protease (Sigma Aldrich, Oakville, ON, Canada) for 1.5 hours followed by 0.3% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) overnight at 37°C. Cells were then resuspended in DMEM supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT, USA), seeded in monolayer at three cell densities (1.7, 4.2, or 8.0×10^4 cells/cm^2), and grown for various times up to 3 weeks. To maintain the same cell number, but vary the density, 1.6×10^4 cells were seeded in wells with three different surface areas (9.6, 3.8, and 2 cm^2). As a negative control, NP cells were isolated, seeded (8.0×10^4 cells/cm^2, surface area 2 cm^2), and grown in monolayer culture under the same conditions as AF cells.

For cell passaging experiments, primary AF cells, isolated from immature (6- to 9-month-old) calves, were seeded (1.7×10^4 cell/cm^2) in DMEM supplemented with 5% FBS and grown in monolayer culture for 7 days. Cells were harvested with 0.5% trypsin-ethylenediaminetetracetic acid (EDTA, Invitrogen, Carlsbad, CA, USA) at 37°C, seeded in monolayer culture, and grown under the same conditions as previously described.

Quantification of the number of cells expressing ALPa

Annulus fibrosus cells in culture were washed, fixed in 10% neutral buffered formalin, and stained with Vector red alkaline phosphatase substrate kit I (Vector Lab, Burlingame, CA, USA) according to the manufacturer's directions for 15 minutes. Nuclei of cells were stained with 4',6-diamidino-2'-phenylindole (DAPI [1 μg/mL in phosphate-buffered saline (PBS)]; Pierce Biotechnology, Inc., Rockford, IL, USA) for 5 minutes. Cells were visualized using a fluorescent microscope. To quantify the number of cells expressing ALPa, between 100 and 150 cells/well were counted in randomly selected areas and the number of ALP-positive cells was expressed as a percent of the total number of cells. Nucleus pulposus cells were used as a negative control.

Quantification of the amount of ALPa

The AF cells after cell isolation and at 1, 2, and 3 weeks of culture were harvested with 0.5% trypsin-EDTA at 37°C for 5 minutes, resuspended into buffer A (0.1% Triton X, 0.2 M Tris HCl, pH7.4, and 45.7 mM NaCl), and freezed/thawed three times to lyse the cells. The solution was clarified by centrifugation at 2,800 rpm for 20 minutes and 4°C and then stored at −20°C until used. Alkaline phosphatase activity was determined by mixing aliquots of the extracts with 0.02 M solution of *p*-nitrophenol phosphate (Sigma Aldrich) in 0.2 M Tris buffer at pH 9.3 for 1 hour at 37°C. The reaction was stopped by the addition of 50 μL of 1.5 N NaOH and quantified spectrophotometrically at 405 nm (Titertek Multiskan; Thermo Fischer Scientific, Nepean, ON, Canada). *p*-Nitrophenol was used to generate the standard curve. Results were normalized to DNA content. Nucleus pulposus cells and deep zone articular chondrocytes (DZAC) were used as negative and positive controls, respectively.

DNA content

Cultures were harvested at various times and digested with papain, and the DNA content was measured with