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Effect of Lyso-phosphatidylcholine and Schnurri-3 on Osteogenic Transdifferentiation of Vascular Smooth Muscle Cells to Calcifying Vascular Cells in 3D Culture

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ABSTRACT

Background: In vitro cell culture is a widely used technique for investigating a range of processes such as stem cell behavior, regenerative medicine, tissue engineering, and drug discovery. Conventional cell culture is performed in Petri dishes or flasks where cells typically attach to a flat glass or plastic surface as a cell monolayer. However, 2D cell monolayers do not provide a satisfactory representation of in vivo conditions. A 3D culture could be a much better system for representing the conditions that prevail in vivo.
Methods and results: To simulate 3D conditions, vascular smooth muscle cells (VSMCs) were loaded with gold–polyvmer–iron oxide hydrogel, enabling levitation of the cells by using spatially varying magnetic fields. These magnetically levitated 3D cultures appeared as freely suspended, clustered cells which proliferated 3–4 times faster than cells in conventional 2D cultures. When the levitated cells were treated with 10 nM lysophosphatidylcholine (LPC), for 3 days, cell clusters exhibited translucent extensions/rods 60–80 μm wide and 200–250 μm long. When 0.5 μg/μl Schnurri-3 was added to the culture containing LPC, these extensions were smaller or absent. When excited with 590–650 nm light, these extensions emitted intrinsic fluorescence at >667 nm. When the 3D cultures were treated with a fluorescent probe specific for calcium hydroxyapatite (FITC-HABP-19), the cell extensions/rods emitted intensely at 518 nm, the λ_{max} for FITC emission. Pellets of cells treated with LPC were more enriched in calcium, phosphate, and glycosaminoglycans than cells treated with LPC and Schnurri-3.
Conclusions: In 3D cultures, VSMCs grow more rapidly and form larger calcification clusters than cells in 2D cultures. Transdifferentiation of VSMC into calcifying vascular cells is enhanced by LPC and attenuated by Schnurri-3.
General significance: The formation of calcified structures in 3D VSMC cultures suggests that similar structures may be formed in vivo.

1. Introduction

In recent years there has been an increasing awareness that 2D cell cultures do not adequately simulate the in vivo condition. This awareness has stimulated a search for new 3D culture methods that mimic this condition. In the period 1997–2005 about 300 papers dealing with 3D cell culture were published and in 2005–2012 ~2000 papers according to PubMed were published. Most of the 3D methods use uniform cell spheroids, a microtiter plate containing uniform histoids, and scaffolds such as hydrogel sponges through which cells can communicate, connect, and move. A significant exception is the 3D culture method using magnetic levitation methodology (MLM). This method allows much faster cell growth and hence performing more experiments per unit time than 2D cultures. MLM has been used successfully with primary lung cells, glioblastoma cells, HUVEC cells, neural stem cells, liver cells, and breast cells. Magnetic levitation culturing is a much improved in vitro cell culturing platform, producing cells that may recapitulate in vivo tissue properties [4]. The applicability of the method to our VSMC system and the accelerated growth rate were important rationales for using MLM in this study. Significantly, cells grown in both cultures produced hydroxyapatite. There is significant data supporting the view that cells cultured in 2D do retain their phenotypes when grown in 3D (please see Ref [4]). For example glioblastoma cells cultured under 2D and 3D conditions exhibit very similar nuclear and N-cadherin staining patterns.

In previous studies we have demonstrated that when 2D cultures of vascular smooth muscle cells (VSMCs) were treated

Abbreviations: CHA, calcium hydroxyapatite; LPC, lysophosphatidylcholine; VSMC, vascular smooth muscle cell; CVC, calcifying vascular cell; HABP-19, hydroxyapatite binding peptide containing 19 amino acids; CEA, carotid endarterectomy; Ca/P, calcium/phosphorus; FBS, fetal bovine serum; Shn3, Schnurri-3; PO, phosphate; GAG, glycosaminoglycan; FITC, fluoroscein isothiocyanate; Gla γE, gamma-carboxy glutamic acid; βA, beta alanine; MLM, magnetic levitation

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12–14 days with 10 nM lyso-phosphatidylcholine (LPC), they underwent transdifferentiation to calcifying vascular cells (CVCs) which form extracellular ridges and extensions containing calcification as indicated by von Kossa and Alizarin Red reagents [1,13–15]. The calcification cascade is controlled by Runx-2, a master transcription factor that modulates the expression of downstream osteogenic proteins. Schnurri-3 has been identified as an inhibitor of Runx-2 in bone differentiation [2,3], suggesting a novel mechanism for controlling the osteoblastic activity of VSMCs in cell cultures. CVCs growing in 2D cultures form extracellular calcified ridges and rods. However, these may not be the most abundant morphologies in 3D calcified tissue. Recently, magnetite beads (*n3D Biosciences*, Houston, TX) have been used to magnetize cells and levitate them in 3D cultures. These 3D cultures grow 3–4 times more rapidly than 2D cultures [4,5], facilitating a detailed study of CVC formation of calcified structures such as nodules and rods.

Recently, we have synthesized a peptide (HABP-19) derived from the α1 region of osteocalcin, having high affinity and specificity for calcium hydroxyapatite. This peptide contains the amino acid sequence βAγEPRRγEVAγELγEPRRγEVAγEL, with the capability of coupling to a reporter group (e.g., FITC, Cy5.5, etc.) bound to the N-terminus. Its Gla residues enable the peptide to bind to CHA but not to other calcium compounds such as calcium oxalate, calcium carbonate, or calcium pyrophosphate. In this study we have used a fluorescein-conjugated peptide (FITC-HABP-19) to visualize CHA in CVC cultures and to monitor calcification in them [6].

2. Materials and methods

2.1. Tissue processing

Carotid endarterectomy (CEA) tissues were resected from patients undergoing unilateral endarterectomy [7–9]. CEA tissues were collected under a protocol approved by the institutional review board for human research at Baylor College of Medicine. Resected tissues were placed in PBS/glycerol (50/50, v/v) and stored at −20 °C until used.

2.2. Total protein extraction

Cells or calcified tissues were rinsed with cold PBS (3×) and lysed or homogenized with a buffer containing 0.1% octylglucoside plus Ripa Buffer (50/50) (*Thermo Scientific*). The lysate or homogenate was then sonicated for 2 min using an Aquasonic 150HT (*VWR*). Total protein was quantified using the BCA Protein Assay (*Thermo Scientific*, Rockford, IL), per manufacturer's instructions and by Nanodrop™ spectrometry (*Thermo Scientific*).

2.3. Isolation of Schnurri-3

A customized antibody (*GenScript*, Piscataway, NJ) was raised against a synthetic peptide (Shn3-Pep) corresponding to a predicted Schnurri-3 epitope (EEAHKKERKPQKPGKYIC). Anti-Shn3-Pep was isolated from crude antisera by chromatography over Sepharose-Shn3-Pep. The antibody was then coupled to Sepharose to prepare an immunoaffinity column for adsorption of Schnurri-3 protein from the total protein extract from normal regions of human common carotid segments obtained by carotid endarterectomy (CEA).

2.4. Regulation of Schnurri-3

In a separate 2D study we have designed and prepared three siRNAs which provided knockdown efficiencies of 96.7%, 66.2%, and 94.5%. The knockdown of Schnurri-3 was accompanied by significant upregulation of RUNX2, a master transcriptional regulator of osteoblast differentiation, resulting in robust upregulation of osteogenic gene expression (e.g. osteocalcin, collagen, osteopontin, alkaline phosphatase, and osteoprotegerin), (K.C. Vickers, Ph.D. Thesis, 2008, Baylor College of Medicine). An expanded description of that study is the subject of a separate manuscript.

2.5. Magnetic levitation of VSMC 3D cultures

Human aortic smooth muscle cells (PH35405A, *Genlantis*, San Diego, CA) were grown in T-175 flasks. When cells reached ~80% confluency they were transferred to 35 × 10 mm petri dishes. During day zero, cells were treated overnight with MagPLL™, a polylysine-based hydrogel containing gold and magnetite nanoparticles (*n3D Biosciences*, Houston, TX). After cells ingested magnetite nanoparticles they were magnetically levitated and transferred to 3D cultures using the Bio-Assembler neodymium magnet (*n3D Biosciences*, Houston, TX). Cell viability was determined by the method of Allison et al. [26].

2.6. Determination of calcium, phosphate, and glycosaminoglycans

Calcium was determined by the Arsenazo III reagent (*Pointe Scientific Inc.*, Canton, MI) [1]. Phosphate was measured using Malachite Green (*AnaSpec*, Fremont, CA) [1]; glycosaminoglycan (GAG) (10–12) was quantified by the Blyscan Assay (*Biocolor*, Carrickfergus, UK), according to the manufacturers' instructions. Staining of cell cultures for GAG was performed with Alcian blue reagent (1 g of Alcian blue in 100 ml of 3% acetic acid, pH 2.5). The cell clusters were exposed to the reagent solution for 30 min, then washed in running water for 2 min.

2.7. Inverted microscopy

A Nikon TMS-F inverted microscope was used to acquire cell culture images during four consecutive days in order to document the transformation of VSMCs from 2D to 3D CVC cultures. The day at which the VSMCs had grown to ~80% confluency was designated as day 0.

2.8. Fluorescence detection

2.8.1. On slides

Fluorescence was detected using the inverted microscope present in the laser capture microdissection instrument (Veritas Microdissection Instrument 704, *Arcturus Biosciences Inc*, CA) equipped with a red fluorescence filter cube (Ex/Em 590–650 nm/>667 nm), a green fluorescence filter cube (Ex/Em 503–547/>565 nm), a blue fluorescence filter cube (Ex/Em 455–495/>510 nm), and a UV fluorescence filter (Ex/Em 340–390/>410 nm). Veritas 2.3 software from the same manufacturer was used. Pellets from each treatment experiment were deposited over pre-cleaned microscope slides (Fisherbrand Superfrost Plus, *Fisher Scientific*, USA).

2.8.2. In microtiter plates

Fluorescence was detected with a Synergy Mx microplate reader using the software Gen5 v1.10.8 (*BioTek*, Winooski, VT).

2.9. Experimental design

The VSMCs were grown in triplicate 3D cultures for each of the following conditions: i) in normal DMEM media + 15% FBS as a negative control; ii) in DMEM media containing 10 nM LPC as a positive control to promote calcification; and iii) in DMEM media containing 10 nM LPC plus 0.5 μg/μl of Schnurri-3 to attenuate calcification.[1].

2.10. Cell viability

VSMC viability was measured with the LIVE/DEAD cell assay described by Alison et al. [27]. A major fraction of cells (~75%) exhibited green fluorescence indicating predominant viability; a minor fraction of the cells (25%) exhibited red fluorescence, indicating cell death.