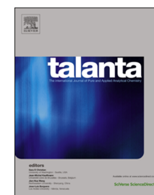
Talanta 130 (2014) 39–48

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta



Discrimination of micromass-induced chondrocytes from human mesenchymal stem cells by focal plane array-Fourier transform infrared microspectroscopy

Chirapond Chonanant [a,1], Keith R. Bambery [b,1], Nichada Jearanaikoon [c], Sirinart Chio-Srichan [c], Temduang Limpaiboon [d], Mark J. Tobin [b], Philip Heraud [e,f,*], Patcharee Jearanaikoon [d,**]

[a] Department of Medical Technology, Faculty of Allied Health Sciences, Burapha University, Chonburi 20131, Thailand
[b] Australian Synchrotron, Clayton, VIC 3800, Australia
[c] Synchrotron Light Research Institute, Public Organization, Nakhon Ratchasima 30000, Thailand
[d] Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand
[e] Centre for Biospectroscopy, School of Chemistry, Monash University, Clayton, VIC 3800, Australia
[f] Department of Anatomy and Developmental Biology, Monash University, Clayton, VIC 3800, Australia

ARTICLE INFO

Article history:
Received 24 February 2014
Received in revised form
19 May 2014
Accepted 20 May 2014
Available online 27 June 2014

Keywords:
Human mesenchymal stem cells
Micromass culture technique
Chondrogenic differentiation
FPA-FTIR microspectroscopy

ABSTRACT

Rapid and sensitive methods for identifying stem cell differentiation state are required for facilitating future stem cell therapies. We aimed to evaluate the capability of focal plane array-Fourier transform infrared (FPA-FTIR) microspectroscopy for characterising the differentiation of chondrocytes from human mesenchymal stem cells (hMSCs). Successful induction was validated by reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis for collagen and aggrecan expression as chondrocyte markers in parallel with the spectroscopy. Spectra derived from chondrocyte-induced cells revealed strong IR absorbance bands attributed to collagen near 1338 and 1234 cm^{-1} and proteoglycan at 1245 and 1175–960 cm^{-1} compared to the non-induced cells. In addition, spectra from control and induced cells are segregated into separate clusters in partial least squares discriminant analysis score plots at the very early stages of induction and discrimination of an independent set of validation spectra with 100% accuracy. The predominant bands responsible for this discrimination were associated with collagen and aggrecan protein concordant with those obtained from RT-PCR and Western blot techniques. Our findings support the capability of FPA-FTIR microspectroscopy as a label-free tool for stem cell characterization allowing rapid and sensitive detection of macromolecular changes during chondrogenic differentiation.

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1. Introduction

Human mesenchymal stem cells (hMSCs) with multiple differentiation potentials are preferred as the predominant cell sources for cartilage tissue engineering [1]. To date, most of the standard protocols for determining stem cell differentiation are laborious and involve cell isolation, fixation and subsequent specific staining. Specialized expensive reagents, as well as stem cell samples, are consumed during multiple testing procedures. In addition, individual sample preparation can be evaluated for only one or two markers at a time. Taken together, these conventional methods are complicated and impractical for the routine analysis of differentiated MSCs destined to be used in clinical practice. Fourier transform infrared (FTIR) microspectroscopy offers the possibility to replace standard methods in stem cell studies. Indeed, previous work has demonstrated the ability of FTIR spectroscopy to detect cells differentiated from stem cells both in vitro [2] and in vivo [3]. As a labeled free technique, FTIR microspectroscopy allows the derivation of a biochemical fingerprint of macromolecules in the cell samples that variously absorb in the mid-IR spectral region. The unique characteristics of IR absorption bands are represented

* Corresponding author at: Centre for Biospectroscopy, School of Chemistry, Monash University, Clayton, VIC 3800, Australia. Tel./fax: +61 3 9905 4597.
** Corresponding author at: Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Kaen40002, Thailand. Tel./fax: +66 4334 7482.
E-mail addresses: phil.heraud@monash.edu (P. Heraud), patjea@kku.ac.th (P. Jearanaikoon).

[1] These two authors contributed equally to this work.

http://dx.doi.org/10.1016/j.talanta.2014.05.037
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for proteins (amide I band at $\sim$1650 cm^{-1}; amide II at $\sim$1545 cm^{-1}; and amide III at $\sim$1300 cm^{-1}), lipid (CH stretching bands at 3000–2800 cm^{-1}; CO stretching from ester groups at $\sim$1730 cm^{-1}) and nucleic acids (asymmetric PO_2^- stretching from nucleic acids at $\sim$1240 cm^{-1}; symmetric PO_2^- stretching from nucleic acids at $\sim$1080 cm^{-1}) [4]. Therefore, FTIR microspectroscopy enables the probing of differences or changes in cellular structures or states.

Although various cell culture methods such as pellet culture, monolayer culture and biomaterial-based scaffold culture have been developed, cell density of induced chondrocytes are still limited [5–9]. Pellet cultures are normally performed in 15 ml polypropylene conical tubes which occupy much incubator space. Change of medium in this system would also be laborious and costly. Furthermore, a number of published papers reported that a tightly aggregated pellet prepared by centrifugation often creates undifferentiated or necrotized cells in the central region of the pellet and only the peripheral cells underwent chondrogenic differentiation [10–14]. Thus, pellet culture is limited for use in clinical applications. Recently, the micromass culture system developed by Scharstuhl et al. [15] was demonstrated to be superior to the pellet culture system in terms of the yield of viable, differentiated cells. Moreover, the micromass system allows spontaneous cell aggregation, and the loose packing of cells compared with cell pellet culture improves diffusion of nutrients and growth factors. Accordingly, higher expression of chondrocyte markers was found in micromass culture compared to that in pellet cultures [12]. Hence, the micromass culture system exhibits more potential for chondrogenic induction.

Synchrotron sources enable an increase in sensitivity by generating more brilliant IR than conventional sources, allowing good signal-to-noise ratio (*S*/*N*) measurement at very small apertures, allowing the best spatial resolution to be achieved [16–19]. However, application of synchrotron-based FTIR microspectroscopy to biomedical research has been limited due to the restricted availability of synchrotron sources. The FTIR spectrometer coupled with a FPA detector operated in the so-called wide-field or global imaging mode is becoming more advantageous in terms of rapid data acquisition time and ease of use compared with synchrotron-based measurement systems. Indeed, the study by Heraud et al. [20] showed that FPA-FTIR microspectroscopy can produce a similar result to a synchrotron-based method when FPA images and synchrotron maps of biological tissue were compared. FPA-FTIR microspectroscopy has also been reported for characterization of various types of human cells and tissues, including brain [21], colon [22], cervix [23], lung [24] as well as stem cell study [4,25,26]. From a previous study, we clearly reported the capability of synchrotron radiation-FTIR to detect differentiation of chondrocyte-induced hMSCs. In this study, we evaluated for the first time the capability of FPA-FTIR microspectroscopy to discriminate the differentiation state of micromass-induced chondrocytes from hMSCs under growth factors (TGF-β3 and BMP-6) supplement for 7, 14 and 21 days. The changes observed in FTIR absorbance bands attributed to collagen (amide III band at 1338 cm^{-1} and the P–O stretching band at 1234 cm^{-1}) as well as proteoglycan (S–O stretching band at 1245 cm^{-1} and C–O–C stretching bands from carbohydrate at 1175–960 cm^{-1}) were used as indicators to monitor chondrocyte differentiation from hMSCs [17,27].

2. Experimental section

2.1. Chondrogenic induction using micromass culture

Human bone marrow mesenchymal stem cells (hBMSCs) purchased from Cambrex Bio Science, Walkerville, MD (cat no. PT-2501) were subjected to chondrogenic induction using micromass culture system, according to the protocol by Mello and Tuan [28]. In brief, hBMSCs were harvested and resuspended in Dulbecco's modified Eagle's medium with a low glucose concentration (DMEM-LG; Gibco Invitrogen) containing 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco Invitrogen) and 10% fetal bovine serum (HyClone, Cramlington, UK) to achieve a final cell suspension of 4×10^6 cells ml^{-1}. To establish micromass cultures, 10 μl drops of cell suspension were spotted in each well of a 24-well plate. After spotting with cells, the plate was pre-incubated for 2 h at 37 °C to permit cell attachment, followed by addition of 300 μl of differentiation medium consisting of DMEM-high glucose with L-glutamine, sodium pyruvate, and pyridoxine hydrochloride (Gibco Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco Invitrogen), 50 μg/ml L-ascorbicacid-2-phosphate (Sigma-Aldrich, St Loius, MO), 0.4 mM L-proline (Sigma-Aldrich), 10^{-7} M dexamethasone (Sigma-Aldrich), 1% ITS+1 (Sigma-Aldrich), 250 ng/ml of BMP-6 (Sigma-Aldrich) and 10 ng/ml of TGF-β3 (Sigma-Aldrich). The medium was replaced every 3–4 days for 3 weeks. Control cells were cultured in parallel without additional growth factors. Three independent experiments were carried out to minimize the change in culture condition. Cells were collected at 7, 14 and 21 days for RNA and protein extraction (Fig. 1).

2.2. Detection of mRNA expression of chondrocyte markers by RT-PCR

Control and chondrocyte-induced cells at day 7, 14 and 21 were subjected to mRNA extraction using RNeasy mini kit (Qiagen, Hilden, Germany), based on the manufacturer's instructions. Approximately 1 μg of total RNA was then converted to cDNA. RT-PCR of three specific chondrocyte marker genes; collagen type II (*col II*), SRY (sex determining region *Y*)-box 9 (*SOX9*) and aggrecan (*AGC*) was performed to verify the successful induction of chondrogenesis. The amplification was carried out in a Thermal Cycler (Corbett Life Science, Australia). The following primers were used: *col II*: 5′-CAG AAG ACC TCA CGC CTC-3′, 5′-TAG TTT CCT GCC TCT GCC TTG AC-3′; *SOX9*: 5′-TGA AGA AGG AGA GCG AGG AG-3′, 5′-GCG GCT GGT ACT TGT AAT CC-3′; *AGC*: 5′-CAG GTG AAG ACT TTG TGG ACA TCC-3′, 5′-CCT CCT CAA AGG TCA GCG AGT AGC-3′; and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): 5′-GTC AGT GGT GGA CCT GAC CT-3′, 5′-AGG GGA GAT TCA GTG TGG TG-3′. The reaction mixture was incubated initially at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 54–60 °C for 40 s and extension at 72 °C for 60 s. The amplified product was separated by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized under an UV trans-illuminator.

2.3. Detection of chondrocyte protein markers by Western blot analysis

Cells were collected at the indicated time points and lysed with 100 μl of protein extraction buffer containing 10 M Tris HCl pH 8.0, 2 M phenylmethanesulfonylfluoride (PMSF) and 1× phosphatase inhibitor mixture (Roche, Mannheim, Germany). Lysates were incubated on ice for 30 min, homogenized, and centrifuged at 13,000 × *g* for 15 min. The supernatant was collected, and protein concentrations were determined by using the Lowry assay. Equal amounts of protein extracts ($\sim$30 μg) were fractionated by 7.5–12% SDS-PAGE and transferred onto PVDF membranes (Amersham Biosciences). Blots were probed at 4 °C overnight with mouse monoclonal anti-human collagen type II (Chemicon International, Temecula, USA) and mouse monoclonal anti-human cartilage proteoglycan (Chemicon International) at a dilution of

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