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Non linear optical microscopy of adipose-derived stem cells induced towards osteoblasts and adipocytes

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Abstract

Adipose-derived stem cells (ADSCs) are adult stem cells isolated from lipoaspirates. They are a good candidate for autologous cell therapy and tissue engineering. For these applications, label-free imaging could be critical to assess noninvasively the efficiency of stem cell (SC) differentiation. We report on the development and application of a multimodal microscope to monitor and quantify ADSC differentiation into osteoblasts and adipocytes.

Keywords

NLO; Multiphoton microscopy; CARS; SHG; live cell imaging; Stem cells; Osteoblasts; Adipocytes

1. INTRODUCTION

Adipose derived stem cells (ADSCs) are adherent adult stem cells with similar properties to mesenchymal stem cells¹. They are, relatively easily, isolated from lipoaspirates, and as such represent a source of autologous stem cells for regenerative medicine. Likely applications include bone, cartilage and adipose tissue reconstruction through cell therapy and tissue engineering.

There are many biological assays to assess the osteogenic and adipogenic potential of adult stem cells. ADSCs can be cultured for few weeks until end point functional assays can be performed such as alizarin red to test for mineralized matrix formation, or Oil-red-O for lipid droplets formation. Earlier signs of differentiation can be tracked by immunohistochemistry staining, FACS, or RT-PCR². These assays are central in regenerative medicine to assess the differentiated state and to assess the efficiency of differentiation. However, they are destructive time-point assays. The field could benefit from additional label-free imaging techniques that will allow time course studies, and noninvasive assessment of the state of differentiation.

Vibrational spectroscopic techniques are potential tools for non-invasive characterization of live cells and tissues. Raman spectroscopy (RS) is a vibrational spectroscopic technique that can be used to optically probe the molecular changes associated with biochemical processes in biological organisms. RS of cells and tissues delivers great information on cell viability, disease and biochemistry. However, RS suffers from a major drawback; inefficiency due to

low signal levels resulting in long acquisition times for microscopy. Such long acquisition times prevent real time imaging of live cells.

Coherent anti-Stokes Raman scattering (CARS) microscopy³ is a nonlinear process in which molecular vibrations are driven coherently through stimulated excitation by pulsed lasers. It offers new possibilities of chemically selective imaging with high sensitivity and three-dimensional capabilities. CARS microscopy gets around the inefficiency of Raman spectroscopy by enhancing the signal level by 4–6 orders of magnitude.^{4, 5} This brings the image acquisition time down to seconds, so biochemical reactions in live cells can be followed in real time. Because the contrast is based on endogenous molecules, and very high sensitivity approaching 10^{-3} M can be achieved, CARS microscopy is recognised as a powerful label-free imaging technique of fluorescence-free biological specimens.^{6, 7}

The combination of CARS and other multi-photon imaging techniques such as two photon fluorescence (TPF), second harmonic generation (SHG) and so on, provides a wealth of chemical and biological information and helps to address the most persistent biological questions.⁸ The multimodal imaging approach has a great potential to contribute to diagnosis of diseases like cancer^{9, 10}, atherosclerosis¹¹ and neurodegeneration¹².

In this study, we investigated multimodal microscopy to monitor and quantify non-invasively ADSCs differentiation into the osteoblast and adipocyte lineages.

2. MATERIALS AND METHODS

2.1 cell culture

Human adipose derived stem cells (ADSCs) (Invitrogen) were expanded to passage 3 in a low serum medium (complete RS MesenPRO, Invitrogen) with 2mM L-glutamine supplement according to the supplier protocol. Cells were cultured in glass bottom petridishes (Fluorodish Cell Culture Dish - 35mm, World Precision Instruments) in a humidified incubator at 37° and 5% CO₂. After ADSCs reached 80% confluency, cells were induced either towards the osteoblast and adipocyte lineages with respectively an osteogenesis and or adipogenesis differentiation medium (Invitrogen). Two weeks post-induction end-point staining was performed to assess that ADSCs were induced towards osteoblasts (.Alizarin red staining), and towards adipocytes (Oil Red O staining).

2.2 Multimodal microscopy

A mode-locked Nd : YVO₄ laser source (PicoTrain, High-Q laser) produces the Stokes pulse (6 ps, 1064 nm) and a 5-ps, frequency-doubled, 532-nm beam, which was used to pump a picosecond optical parametric oscillator (OPO) (Levante Emerald). The OPO delivers a signal tuneable in the range 700–1000 nm, which was used as a pump in the CARS process. The two beams are combined in a collinear geometry and focused on the sample using a 60× oil immersion objective (Plan Apo VC, NIKON) with 1.4 numerical aperture (NA). A laser-scanning confocal inverted optical microscope (Nikon BV 'C1', Amsterdam, Netherlands) is used to acquire images. The configuration of the system enables both backward (epi-) and forward detection schemes. Also, TPF and SHG images can be carried out at the same time as CARS using a three channels detection scheme. An appropriate set of dichroic mirrors, short-pass and band-pass filters (Chroma, Rockingham, USA) are used to selectively transmit the NLO signals. The laser power at sample was 12 mW and 8 mW for the pump and Stokes beams, respectively. All images are (212 μm × 212 μm) and (512 × 512) pixels. A schematic diagram of our multimodal microscope is presented in figure 1.

3. RESULTS AND DISCUSSION

3.1. CARS imaging of adipo-induced cells

ADSCs induced towards adipocytes are characterised by the presence of intracellular lipid droplets (LDs) accumulation, mainly around the nucleus. To monitor the differentiation of ADSCs cells to fat cells, live CARS images were taken at different stages of differentiation.

CARS imaging was performed by tuning the Stokes (1064 nm) and pump (817 nm) beams to excite the Raman peak at 2845 cm^{-1} (663nm) corresponding to the CH_2 stretch in lipids. Obtained CARS images and their corresponding transmitted light images after 2, 7 and 10 days post-induction are presented in figure 2. Images of undifferentiated ADSCs cells are presented as a control.

Two days after induction, many small bright spots corresponding to LDs appeared near the periphery of the nuclei (Fig. 2B) in adipo-induced cells. Moreover, a change of morphology to a more rounded shape, and an increase of the mean cell diameter are observed for cells undergoing adipogenesis. Figures (2-c, 2-d) show the accumulation of LDs. After 7 days, LDs became bigger and well structured around the nuclei. Their size appears to increase continuously (to reach 20 to 25 μm) until they completely fill the cytoplasm surrounding the nuclei as shown in (Fig. 2-D) after 10 days. Identification of these features was confirmed by Oil red O staining (data not shown). These study shows that early signs of differentiation of ADSCs to adipocytes can be observed with label-free CARS imaging. Similarly the efficiency of differentiation could be easily calculated as the ratio of differentiated cells over to undifferentiated cells.

4.2. CARS imaging of osteo-induced cells

Osteo-induced cells are characterized mainly by hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (HA)¹³ mineralization - a precursor to bone - and the formation of an external cellular matrix of collagen.¹⁴

Figure 3 shows CARS imaging of cell membrane obtained at 2845 cm^{-1} and their corresponding light transmitted images. Changes in cell morphology of osteo-induced cells were observed the 2nd day after induction. Osteo-induced cells became longer in shape and thinner than ADSCs Cells induced towards osteoblasts covered more effectively the substrate as compared to adipo-induced cells.

Three dimensional CARS images show that cells undergoing osteogenesis were growing in multiple layers (Fig. 4). Such important information cannot be obtained from light transmitted images.

Typically the first signs of mineralization appeared between the 10th and 15th days as confirmed by Alizarin red staining used to validate the osteoblast lineages. HA mineralization could be quantified with CARS imaging to characterize the ADSCs differentiation towards the osteogenesis. To do so, Stokes and pump beams have to be tuned to excite the characteristic Raman peak relating to HA which is produced in the extracellular matrix and carbonate observed 960 cm^{-1} and 1067 cm^{-1} respectively.¹⁵

Similarly nuclei imaging with CARS at the DNA frequency (1095 cm^{-1})¹⁶ could be performed to facilitate cells and counting and the assessment of the efficiency of differentiation as the ratio number of cells to quantification of mineralization.

CARS wavelength corresponding to these frequencies are 863.6 nm (DNA), 867.4 nm (HA) and 883.8 nm (carbonate). Our microscope is equipped with photodetectors which are less

sensitive to these wavelengths. Thus high laser power and long time acquisition are needed to image the DNA. In these conditions, a photodamage is can occur easily, and CARS microscopy will be invasive. In order to overcome this technical problem, more developments are required like using more sensitive detectors or other techniques like stimulated Raman spectroscopy (SRS).¹⁷

4.3. Multimodal microscopy imaging of osteo-induced cells

Multiphoton microscopy allows noninvasive and stain free imaging of cells and tissues using endogenous contrast through TPEF and SHG. TPEF has been used to characterise cell viability, morphology and proliferation in tissue. Recently, TPEF has enabled researchers to monitor the metabolic activity, morphology and oxidative stress of hMSCs¹⁸. In addition, SHG has been used to monitor fibrous collagen deposition¹⁸. Thus, TPEF and SHG microscopy of ADSCs undergoing osteogenic differentiation can be used as a direct method of characterising cell morphology and the deposition of fibrous collagen.

We acquired TPEF and SHG images simultaneously using a 570 nm dichroic mirror and 414/46 nm (SHG) and 609/54 nm (TPEF) band-pass filters. Obtained multimodal images are presented in figure 5.

At day 7 post-induction (Fig 5-c), collagen fibres are observed accompanied by an increase of fluorescence of structures surrounding the nuclei (Fig 5-b). This fluorescence can be attributed to oxidised flavo-proteins (FP). This is in good agreement with literature suggesting an increase of redox ratio (ratio of reduced pyridine nucleotide (NADH) to FP) upon transition from stem cells to differentiated state¹⁸⁻²⁰. This becomes more evident at day 10 where this fluorescence is stronger and the collagen matrix is denser (Fig 5-e,f).

The composite image CARS/TPEF/SHG presented in figure 6 is rich of information. Among these information, metabolic changes of ADSCs during their differentiation towards the osteoblast lineage can be obtained from TPEF of FP, fibrous collagen deposition from SHG and morphology from CARS imaging. All these crucial information required to monitor ADSCs differentiation cannot be extracted from light transmission or phase contrast images.

CONCLUSION

The induction of ADSCs towards two different cell lineages was monitored in parallel using CARS, TPEF and SHG. Multimodal microscopy showed the potential to monitor and quantify differentiation state with the same efficiency as normal confocal fluorescence microscopy but without using labelling agents. Multiphoton microscopy holds considerable promise for the non-invasive assessment of the differentiation state of SCs prior to their use for cell therapy in regenerative medicine.

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Figure 1. Schematic diagram of Multimodal microscope setup. M, mirror; DM, dichroic mirror; GM, galvanometer; O, objective; S, sample; C, condenser; SPF, short pass filter set; L, focusing lens; MF, multimodal optical fibre.

Figure 2.
CARS images of ADSCs cells (A) induced towards adipocytes at different stages post induction: 2 days (B), 7 days (C) and 10 days (D). Image size $212 \mu\text{m}^2$ and acquisition time was 21s for (a and b) and 1s for (c and d).

Figure 3. CARS images of ADSC cells (A) induced towards osteoblasts at different stages of differentiation. 2 days (B), 7 days (C) and 10 days (d). Image size $212 \mu\text{m}^2$ and acquisition time was 21s for all images.

Figure 4.

Z-stack of 16 images of ADSC cells differentiated towards osteoblasts at day 10 post-induction taken 1 μm steps from the bottom (top left image). The pictures show a multi-layered distribution of the cells. Note the different morphologies of the cells. Larger cells, closer to the real morphology of bone cells can be observed. Image size 212 μm^2 and acquisition time was 21s for all images.

Figure 5. Multimodal images of ADSCs cells induced towards osteoblasts; 7 days (a-c) and 10 days (d-f) after induction: (a,d) CARS, (b, e) TPEF of flavo-proteins, (c, f) SHG of fibrous collagen. Acquisition time was 21s per image. Image size $212 \mu\text{m}^2$ and acquisition time was 21s for all images.

Figure 6. Comparison of multimodal images of ADSCs cells induced towards osteoblasts; 7 days (a-b) and 10 days (c,d) after induction and their transmitted light images. CARS (red), TP of flavo-protein (green) and SHG of collagen (blue). Image size $212 \mu\text{m}^2$ and acquisition time was 21s for all images.