Mechanisms of Artificial Cartilage Generation in Polyglycolic Acid Scaffold Revealed by Multiphoton Microscopy

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Abstract: Multiphoton microscopy is a non-linear excitation imaging technique useful in probing structural and dynamic phenomena in biological systems. In this work, the advantages of multiphoton microscopy is explored to follow the physiological changes to tissue engineered cartilage using human mesenchymal bone marrow stem cells cultured in polyglycolic acid (PGA) scaffold. Multiphoton induced intrinsic luminescence signals from these specimens reveal the generation of extracellular matrix distorts the PGA scaffolds and show that multiphoton microscopy can be a useful technique in assessing the physiological states of tissue engineered constructs.

INTRODUCTION

The development of two-photon fluorescence microscopy in the 1990s revolutionized the way in which optical microscopy is performed in living biological systems. Unlike conventional fluorescence microscopy which uses UV or visible photons to induce single-photon transitions from fluorescent molecules, two-photon microscopy used near-infrared photons to induce the simultaneous absorption of two less energetic photons for fluorescence excitation. A number of significant advantages are associated with this approach. First, since two-photon excitation is a non-linear process with an absorption probability proportional to the square of incident photon flux, the fluorescence generation is limited to the focal volume where the photon flux is high. Images obtained from scanning the confinement of the excitation spot (around 0.1 femtoliter) result in confocal-like imaging quality without using a detection aperture. The localized focal volume also limits the specimen damage to a single point within the sample. Furthermore, since the near-IR photons used for sample excitation are absorbed and scattered less by tissue components than the UV or visible photons in standard fluorescence microscopy, greater imaging depths can be achieved without damaging histological procedures. Finally, the wide spectral separation of the two-photon excitation wavelengths from the sample fluorescence results in a more efficient detection of the sample luminescence signals. These advantages have enabled non-linear excitation microscopy to be useful in addressing key issues in many areas of the biological sciences. Additional developments in non-linear excitation technologies have led to the imaging modalities such as multiphoton microscopy or harmonic generation microscopy. These developments have added to form a complete repertoire of non-linear excitation microscopy.

METHODS AND MATERIALS

Mesenchymal Stem Cell Preparation

Bone marrow from human femurs were obtained and mixed in 1:9 (v/v) ratio with a processing medium composed of 0.8% ammonium chloride and 10 μ M EDTA (Sigma, St. Louis, MO) for 10 minutes. After lysis of the red blood cells, the mixture was centrifuged at 500 g for 10 minutes and resuspended in the culture medium composed of DMEM-low glucose (Gibco, Grand Island, NY), 10% fetal bovine serum (HyClone, Logan, UT), 10 U/ml penicillin G, and 10 μ g/ml streptomycin (Gibco).

Stem Cell Seeding Onto PGA Scaffold and Tissue Induction

Following the procurement of the human femoral bone marrow cells, they are seeded onto polyglycolic acid (PGA) scaffold (Synthecon, Houston, TX). The PGA scaffold were sterilized and cut to an area of 3.5 by 3.5 mm². To see the bone marrow cells, the PGA scaffolds were placed on sterile pads and 20 μ l of the suspended cells (2 $\times$ 10⁷ cells/ml) were placed onto the scaffold. Next, the scaffold was flipped and placed on a 6-well plate before another 20 μ l of the suspended cells were placed on the scaffold. The cell-filled scaffolds were then transferred to a 37°C incubator where over the next 2 hours, 10 μ l of the culture medium was placed onto the scaffold at hourly intervals. For the next 6 hours, 10 μ l of the culture medium were used to exchange the

THP-27(T5)

scaffold medium. Finally, 5 ml of the culture medium were used to immerse the cell-seeded scaffolds for long term culture. To ensure that the seeded cells become firmly attached to the scaffolds, the cell-scaffold composites were left undisturbed for 3 days.

To induce cellular differentiation from the bone marrow cells seeded in the PGA scaffolds, the cell-scaffold cultures were transferred into 24 well plates and replaced with a different culturing medium containing the transforming growth factor $\beta 3$ (TGF- $\beta 3$).

Multiphoton Microscope Instrumentation

The 780 nm output of a diode-pumped (Millennia V, Spectra Physics, Mountain View, CA), titanium-sapphire laser (Tsunami, Spectra Physics) was guided into a commercial laser scanning system (Meta 510, Zeiss, Germany). The excitation source was reflected into the back aperture of the objective using a short-pass dichroic mirror (HFT KP650, Zeiss). The luminescence signal generated at the sample was collected by the objectives (25x, NA 0.8 Imm oil immersion or 40x, NA 1.3 oil immersion, Zeiss) and an additional short-pass filter (KP685, Zeiss) prior to reaching the photomultiplier tube. In this manner, we collect the broad band luminescence (below about 650 nm) from the tissue engineered PGA scaffold for microscopic imaging. The power used was 4 mW at the sample.

RESULTS

Conventional analysis of tissue engineered products have based on histological imaging and biochemical assays of secreted proteins. While these techniques are useful in revealing the biochemical and structural states of engineered tissues, the processes involved often required the fixation of the specimens of interest. The dynamic information present in tissue reorganization processes is absent using these techniques. On the other hand, the minimally invasive nature of multiphoton microscopy enables the visualization of the states of the tissues without histological procedures. As tissue engineering advances, the need to monitor engineered tissues in vitro should prompt researchers to utilize the advantages of multiphoton microscopy.

CONCLUSIONS

In this investigation, we use multiphoton microscopy to image the changes in stem cell physiology following seeding of human mesenchymal bone marrow stem cells into polyglycolic acid (PGA) scaffold. Multiphoton microscopy was able to follow the generation of extracellular matrix and deformation of the PGA scaffold at different times following TGF- $\beta 3$ induction.

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