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Monitoring wound healing of elastic cartilage using multiphoton microscopy

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SUMMARY

Objective: To demonstrate the ability of multiphoton microscopy (MPM) for monitoring wound healing of elastic cartilage.
Method: In a rabbit ear model, four cartilage specimen groups at 1-day, 1-, 4-, 20-week healing time points as well as a normal elastic cartilage were examined with MPM without using labeling agents. MPM images at wound margins were obtained from specimens at different healing stages, compared with the Hematoxylin and Eosin (H&E) stained images. Image analysis was performed to characterize the collagen morphology for quantifying the wound healing progression of elastic cartilage.
Results: MPM provided high-resolution images of elastic cartilage at varying depths. Comparisons of the images of specimens at different healing stages show obvious cell growth and matrix deposition. The results are consistent with the histological results. Moreover, quantitative analysis results show significant alteration in the collagen cavity size or collagen orientation index during wound healing of elastic cartilage, indicating the possibility to act as indicators for monitoring wound healing.
Conclusion: Our results suggested that MPM has the ability to monitor the wound healing progression of elastic cartilage, based on the visualization of cell growth and proliferation and quantitative characterization of collagen morphology during wound healing.

Introduction

Wound healing of elastic cartilages is one of the most important issues of plastic surgery[1–3]. It involves complex processes including growth and proliferation of chondrocytes and deposition of new matrix surrounding the cells. Due to the lack of blood vessels, cartilage grows and repairs more slowly compared to other connective tissues. Numerous attempts are made to develop an efficacious strategy for accelerating cartilage repair. The assessment of the therapy response and efficacy requires continual and timely examination of the wound status, especially visualization and quantification of the changes in cells and matrix for monitoring wound healing. Histopathological examination is evidently the gold-standard approach for evaluating wound healing, but its destructive nature makes it impractical for widespread clinical utilization. It is clearly crucial to develop a minimally invasive imaging modality capable of generating information comparable to histology, allowing for monitoring wound healing and guiding the establishment of novel and efficacious therapies.

Recently, multiphoton microscopy (MPM) based on second harmonic generation (SHG) and two-photon excited fluorescence (TPEF) has been widely used for imaging structure and dynamic interactions in biological tissues[4,5]. The advantages of this technique over other visualizing techniques, such as electron microscopy, magnetic resonance imaging, and confocal microscopy[6–8], include inherent optically sectioning, enhanced penetration depth, and reduced specimen photo-bleaching. SHG enables direct imaging of anisotropic biological structures of collagen by interacting with highly non-centrosymmetric molecular assemblies[9,10]. TPEF is a nonlinear process well suited for high-resolution imaging of intrinsic molecular signals from elastin and cells. The combination of these two mechanisms can provide in tandem complementary information on tissue architecture and function. Moreover, the 3D optical sectioning can provide additional information on the ultrastructure of chondrocytes and the extracellular matrix proteins

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contained within the image stacks[11]. Owing to the ability to generate images based on the natural intrinsic fluorescence properties of tissue, MPM has been used in imaging of articular cartilage and joint synovium for potential diagnosis of inflammatory and degenerative joint diseases without the introduction of exogenous probe molecules or fixation and staining of the tissue[12,13].

To the best of our knowledge, MPM assessment of wound healing of elastic cartilage has not yet been reported, which motivated us to demonstrate the potential of MPM in probing the changes of cells and matrix in healing cartilage. Since rabbits have the capacity to regenerate new tissue for repairing holes in their ears, they are well accepted as a model for histological, biochemical, and biomechanical analysis of *in vitro* and *in vivo* cartilage repair[14,15]. In this study, fresh and unstained cartilage specimens from rabbit external ears at various stages of wound healing were imaged by MPM to investigate the wound healing process of elastic cartilage, compared to the Hematoxylin and Eosin (H&E) histological sections. It is of great significance to image and quantitatively characterize the features of repairing cartilage for confirmation of the newly proposed method under *ex vivo* conditions, before performing *in vivo* investigation for clinical application.

Materials and methods

Specimen preparation

The study design employed five adult New Zealand white rabbits (10 ears) with the approval of the Animal Care and Use Committee, Fujian Medical University. Each ear received two punch wounds of 8 mm diameter from the medial surface extending deep through the cartilage without penetrating the lateral skin. A biopsy specimen was taken from the margin of one wound of each rabbit using a 2 mm diameter biopsy punch 1, 7, 28, 140 days after wound creations. In this manner, four cartilage wound groups at 1-day, 1-, 4-, and 20-week time points (each contains five specimens from five rabbits) were collected. During the punch infliction or biopsy removal, the rabbits weighing 2.0–2.6 kg were anaesthetized with an intravenous injection of 2.5% sodium pentobarbital (2 ml/kg). A schematic drawing of a wound model is shown in Fig. 1 to illustrate the region of interest (ROI), which is marked with dotted lines. Biopsy specimens were cut transversely on a freezing microtome into sections 100 μm thick for MPM imaging and 5 μm for H&E stain. The section for MPM imaging was sandwiched between the microscope slide and cover glass and soaked with a little PBS solution to avoid dehydration or shrinkage during the imaging process.

Histological analysis

The sections of each specimen were processed for histological examination with H&E stains according to standard procedures and reviewed by a certified pathologist. They were imaged using standard bright field light microscope (Eclipse Ci-L, Nikon Instruments Inc., Japan) with a CCD (Nikon, DS-Fi2, Japan). The H&E images from light microscopy (×20) were then compared to MPM images for confirmation of the structures and cells.

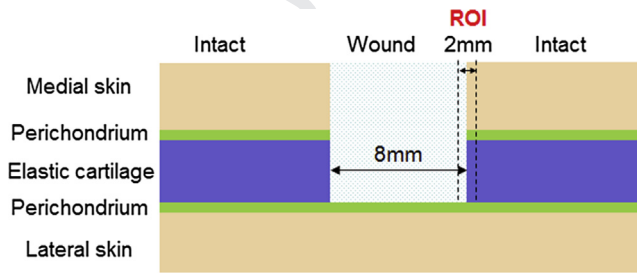

Fig. 1. Schematic illustration of a wound model of elastic cartilage in the rabbit external ear. The region marked with dot lines indicates the ROI.

Multiphoton microscopic system

The MPM system has been described previously[16]. Briefly, a commercial LSM 510 META (Zeiss, Jena, Germany) equipped with a mode-locked femtosecond Ti: sapphire laser (110 fs, 76 MHz) operating at 810 nm (Coherent Mira 900-F) was utilized to obtain high-resolution images of repairing cartilage tissues. A Plan-Neofluar objective (40× and NA = 0.75, Zeiss) was employed for focusing the excitation beam into tissue samples and was also used to collect the backscattered intrinsic SHG and TPEF signals. Two different channels of the META detector were selected to obtain the high contrast images. One channel corresponded to the wavelength range of 398–409 nm to collect collagen SHG signals, whereas the other channel covered the wavelength range of 430–697 nm in order to image the microstructures of cells and elastin. An optional HRZ 200 fine focusing stage (Carl Zeiss) was used to move the motorized x-y scanning stage for obtaining a large-area image and change the focus position for recording various optical sections. Furthermore, 3D images, called z-stacks with 12 sequential 2D images at intervals of 2 μm were obtained for quantitative analysis by sequentially changing the plane of focus (z-level) to scan at varying depths.

Quantification methods

Collagen morphology in auricular elastic cartilage was characterized by collagen cavity size and collagen orientation. Twelve sequential 2D images at intervals of 2 μm were analyzed for averaging. The first morphological characterization, collagen cavity size, was quantified by calculating the averaged cavity area within a stack of SHG images based on an image segmentation algorithm[17]. In detail, from each of the 2D SHG images, binary images separating the collagen from the cavities containing chondrocytes were generated using Otsu's thresholding technique[18], analyzed using ImagePro Plus software (version 6.0). The total number of pixels which was assigned to cavities and the number of cavities within each 2D image were obtained and defined as A_i and n_i, respectively. Then the averaged area of the cavities occupied by chondrocytes was calculated according to the following formula: $\frac{1}{N}\sum_{i=1}^{N} A_i/n_i$, where the index i refers to a frame in the stack and N is the total number of the frames used in the analysis ($N = 12$ in this study)[11].

The second morphological characterization, collagen orientation, was quantified by Fourier analysis method using the Fast Fourier Transform (FFT) module of ImageJ software (version 1.41, NIH)[19]. Fourier analysis has been widely applied to determine the orientation of structures[20]. FFT can represent all frequencies present in an image by using the power plot. Parallel collagen orientation results in an elongated power spectrum whereas randomly organized tissue yields a nearly circular spectrum. Here, the power plot of FFT image was fitted with a 95% confidence ellipse which was performed in Matlab software (version 7.11)[21]. And then, the collagen orientation index defined by $(1 - W/H)$ was used to estimate the orientation extent, where W and H represent the width and height of the ellipse in the generated power plot of the SHG image, respectively. According to the definition, the collagen orientation index approaches zero when the collagen bundles are perfectly randomly oriented, whereas the collagen orientation index approaches "1" when the collagen bundles are organized in an

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