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NON-LINEAR SPECTRAL IMAGING MICROSCOPY STUDIES OF HUMAN HYPERTROPHIC SCAR

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Skin scar is unique to humans, the major significant negative outcome sustained after thermal injuries, traumatic injuries, and surgical procedures. Hypertrophic scar in human skin is investigated using non-linear spectral imaging microscopy. The high contrast images and spectroscopic intensities of collagen and elastic fibers extracted from the spectral imaging of normal skin tissue, and the normal skin near and far away from the hypertrophic scar tissues in a 10-year-old patient case are obtained. The results show that there are apparent differences in the morphological structure and spectral characteristics of collagen and elastic fibers when comparing the normal skin with the hypertrophic scar tissue. These differences can be good indicators to differentiate the normal skin and hypertrophic scar tissue and demonstrate that non-linear spectral imaging microscopy has potential to noninvasively investigate the pathophysiology of human hypertrophic scar.

Keywords: Non-linear spectral imaging microscopy; human hypertrophic scar; collagen and elastin fibers.

1. Introduction

Skin scar is unique to humans, the major significant negative outcome sustained after thermal injuries, traumatic injuries, and surgical procedures. They are hard, pain, itchy, raised and contracted, which probably result in loss of human local function, even disability. And they are also aesthetically ugly, which cannot be cosmetically accepted.[1, 2] Skin scar is usually classified into the normal scar, keloid and hypertrophic scar. Keloid and hypertrophic scars are forms of aberrant wound healing, which are characterized by the overproduction of collagen. At present, there are various clinical treatment methods for hypertrophic scar such as intralesional corticosteroids, laser therapy, surgery, cryotherapy, radiation, and silicone products.[3,4] But the clinical effect is not obvious due to a lack of understanding for pathophysiology of human hypertrophic scar.[5,6] On the other hand, there are no useful animal models that have been developed to produce scars analogous to human hypertrophic scars. This further blocks the investigation of development for pathophysiology of human hypertrophic scar in laboratory and clinical application.[7–9]

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The non-linear spectral imaging technique, which is a combined non-linear imaging approach based on two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG) with the measurement of intrinsic emission spectra of biological tissues, allows simultaneous recording of images and spectra of unstained tissue specimens.[10–12] This method has many advantages over those that only give the autofluorescence spectra or the morphological structure of tissues. It has great potential to provide comprehensive information for the diagnosis and therapy of tissues, and it has been applied to the biochemical analysis of skin tissues in living mouse, excised rabbit esophageal tissues, and excised human skin dermis.[10–12]

In this paper, the characteristics of human hypertrophic scar were studied by using the non-linear spectral imaging technique based on TPEF and SHG. We are trying to discuss if this method has the potential to noninvasively study the pathophysiology of human hypertrophic scar and assess its treatment response in clinic *in vivo.*

2. Materials and Methods

The *ex vivo* human hypertrophic scar specimens were obtained from a 10-year-old patient undergoing skin plastic surgery. An informed consent for study was given by the patient. We strictly conform to the institutional rules governing clinical investigation of human subjects in biomedical research. The biopsy samples were immediately snap-frozen in liquid nitrogen after they were excised from the patient by clinician. The tissue sections for non-linear spectral imaging were cut in 30 μm thickness. Then, they were sandwiched between the microscope slide and coverslip. The cover glass was facing the microscope objective. Moreover, to avoid dehydration or shrinkage during the whole imaging process, a little phosphate buffered saline (PBS) solution was dripped on to the specimen.

The spectral imaging microscopy system used in this study has been described in detail previously.[11] An excitation light source, a high-throughput scanning inverted microscope (Zeiss Axiovert 200), and a spectral imaging detection system (META detector) are the three basic components. The excitation light source (Coherent Mira 900-F) is a mode-locked Ti: sapphire laser with 110fs pulse width and a repetition rate of 76 MHz, which is tunable from 700 nm to 980 nm. The laser intensity is controlled by an Acousto-Optic Modulator (AOM) before entering a Zeiss Axiovert 200 microscope. An oil immersion objective (Plan-Apochromat 63×, N.A.1.4, Zeiss) was employed in all experiments in order to obtain high-resolution imaging. The backward SHG and TPEF signal were collected by the META detector, which comprises a high-quality, reflective grating and an optimized 32-channel photomutiplier tube (PMT) array detector and can acquire a real-color RGB non-linear spectral image.[11,12] All 32 photomultipliers of the META detector cover a spectral width of approximately 340 nm ranging from 377 nm to 716 nm, and a single PMT covers a spectral range of 10.7 nm. In this work, all images were 512 × 512 pixels and have a 12-bit pixel depth. The images were obtained at 2.56 μs per pixel. An extracting channel tool of experimental system was used to obtain high contrast and high-resolution images of collagen and elastic fiber in the hypertrophic scar tissue: one channel covered the wavelength range between 398 nm and 414 nm to show the microstructure of collagen fiber in SHG images, whereas the other channel covered the range from 430 nm to 714 nm in order to image elastic fiber when the excitation wavelength $\lambda_{ex} = 810$ nm was used. Using the image-guide spectral analysis method, the emission spectra of the region of interest within spectral image can be investigated by plotting the mean intensity of all pixels within the region of interest versus the center wavelength of each emission band.

In order to further understand the randomly-orientated arrangement of collagen and elastic fibers in hypertrophic scar tissue, image texture feature analysis method is used to analyze their microstructures. Because both collagen and elastic fiber in human skin each has their special characteristics, common texture features such as co-occurrence matrix and run-length matrix cannot describe them effectively.[13] The edge direction extraction on corresponding images was presented to investigate the distribution patterns of collagen and elastic fibers in normal skin and scar tissues. The gradient information of images is obtained by using Sobel edge detector and applied to Eq. (1) to calculate the directions of every edge pixel.

$$\theta = \arctan\left(\frac{gradY}{gradX}\right) \tag{1}$$

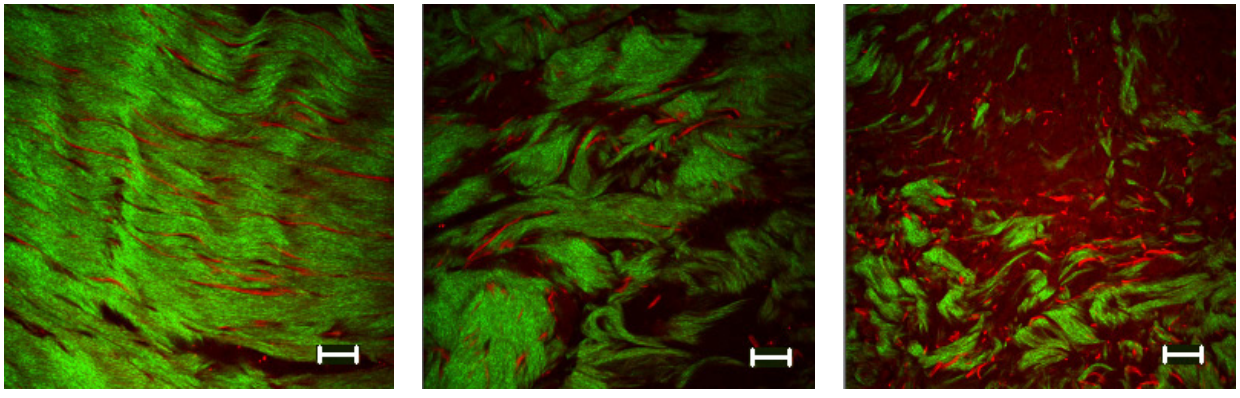
where $gradY$ and $gradX$ are the vertical gradient value and horizontal gradient value, respectively. θ is the direction of edge. Finally, the edge direction histogram of images is obtained.[14]

3. Results and Discussion

It is well known that collagen and elastic fiber are important extracellular matrix elements during tissue repair, which is closely related to the formation of scar. Collagen is a particularly interesting subject for SHG imaging because of its non-centrosymmetric structure.[15,16] If the excitation light source with 110fs pulse width has a full-width at half-maximum (FWHM) bandwidth of 15 nm, the SHG signal spectrum will have a FWHM of $1/\sqrt{2}$ of the fundamental or approximately 10 nm with the peak at precisely half of the excited light wavelength. Elastic fiber is more effective in generating TPEF signal with a broad spectral range at the peak around 500 nm.[16–18] So, the emission spectral range of collagen SHG images and elastin TPEF signal can be completely separated when the infrared femtosecond laser was used as the excitation light source. Using the extracting channel tool of the experimental system, Fig. 1 presents the high contrast images of collagen and elastic fiber extracted from the spectral imaging of normal tissue, and the normal skin near and away from the hypertrophic scar in the 10-year-old patient's case. The extracted spectral range of collagen SHG image is between 398 nm and 414 nm, whereas the elastic fiber TPEF signal is covering the spectral range from 430 nm to 714 nm. The green color-coded structure is collagen and the red color-coded is elastic fiber component in Fig. 1.

It can be seen that collagen consists of a fine mesh morphological structure with well-regulated distribution and orientation in normal skin tissues (in Fig. 1(a)), which is consistent with the results of normal skin dermis without scar disease as reported in Refs. 16 and 18 based on multiphoton microscopy. In contrast, the distributions of collagen in the normal skin near and away from the hypertrophic scar are apparently disorganized, even disrupted and the density of collagen seems much lesser, as shown as in Figs. 1(b) and 1(c). The collagen fibers lie in haphazardly-connected, loose sheets that appear randomly oriented. Especially so, as the collagen fibers are not only disrupted but also obviously organized in swirls in Figs. 1(b) and 1(c), which shows that less collagen SHG image can be achieved. These are consistent with the results of hypertrophic scar tissues based on the method of immunohistochemical staining.[19] And this scar image pattern is similar with the results of human keloid using multiphoton microscopy.[20] The morphological structure of elastic fiber also presents clear difference in normal skin and hypertrophic scar tissue. The elastic fiber in normal tissue displays the morphology of straight ropes which coil and recoil like a spring to ensure skin structures have fine elasticity. However, in the hypertrophic scar tissue, the distribution of elastic fibers is disrupted like that of collagen fibers and elastin is present in fragmented fibers. This is why the hypertrophic scar tissue is usually hard and has no elasticity.

The non-linear spectral imaging technique based on TPEF and SHG can simultaneously acquire images and spectra of unstained tissue specimens, thus providing more accurate and



(a) The normal skin tissue

(b) Hypertrophic scar near normal tissue

(c) Hypertrophic scar away from normal tissue

Fig. 1. High contrast images of collagen and elastic fibers extracted from the spectral imaging of the normal tissue, and the normal skin near and far from the hypertrophic scar tissue in a 10-year-old patient's[2] case. The excitation wavelength λ_{ex} was 810 nm. Scale bar = 20 μm.

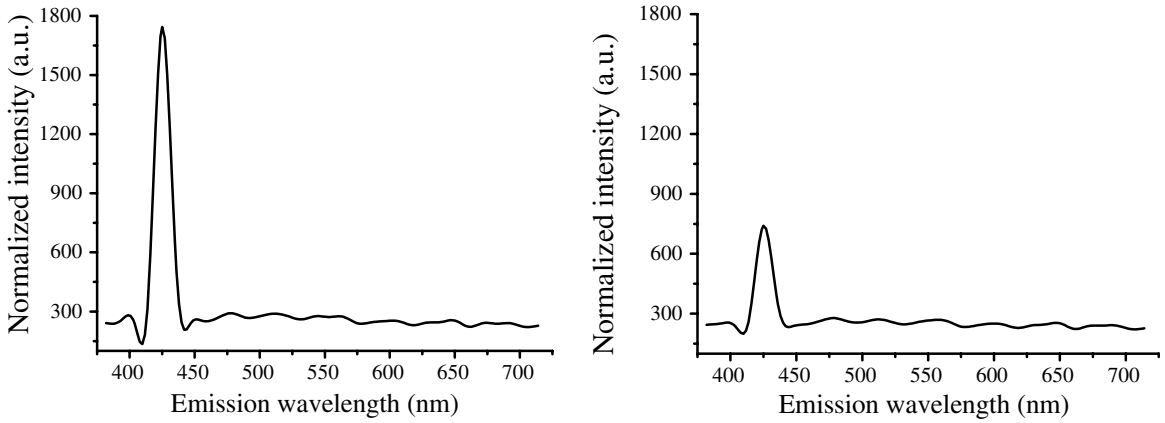


Fig. 2. The emission spectra of normal skin and hypertrophic scar tissue corresponding to the images in Figs. 1(a) and (b) which are extracted from their spectral imaging by using the image-guide spectral analysis method.

comprehensive information for determining tissue physiological and pathological states. The structural difference of collagen in Figs. 1(a) and (b) shows that their spectral intensity presents obvious change. Figure 2 shows the emission spectra of normal skin and the normal skin near the hypertrophic scar corresponding to the images in Figs. 1(a) and (b), which are extracted from their spectral imaging by use of the image-guide spectral analysis method. One can see that the collagen SHG signal intensity in the normal tissue was about 2 times that in the hypertrophic scar tissue near the normal skin (elastin fiber TPEF signal is too weak to be clearly shown in Fig. 2 when compared with collagen SHG signal). This indicates that arrangement of collagen in the normal skin is well regulated than that in the hypertrophic scar tissue. During plastic surgery, a clinician has difficulty judging the exact boundary between normal skin and hypertrophic scar tissues. They usually perform the plastic surgery according to their clinical experience and naked eye observation and hope that all scar tissues can be completely excised from the patients but with minimal removal of normal skin tissue. So, the apparent difference of collagen SHG signal intensity in the normal tissue and the hypertrophic scar tissue near the normal skin can be an indicator to identify and judge the exact boundary between normal skin and hypertrophic scar tissues.

Using image texture feature analysis method, the edge direction histogram of elastic and collagen fibers in normal skin and hypertrophic scar tissues corresponding to Figs. 1(a) and (b) was shown in Fig. 3. The X axis θ represents all direction of edge, and the Y axis n represents the number on the edge direction. From Fig. 3, it can be seen that the edge direction histograms of collagen and elastic fibers in the normal tissue all present the distribution with a shape peak, which means the collagen and elastic fibers in the normal skin are largely orientated in the same direction. Conversely, the edge direction histograms of collagen and elastic fibers in hypertrophic scar tissue have no apparent shape peak distribution, which results in the random nature of fiber orientation.

4. Conclusion

The non-linear spectral imaging technique has the ability to differentiate between the normal skin and hypertrophic scar tissue through the microstructure and spectral features of collagen and elastic fibers based on collagen SHG image and elastin TPEF signal. There are obvious differences in collagen fiber and elastin fiber between normal skin and hypertrophic scar. These differences will be good indicators to distinguish the normal skin from skin scar or to evaluate clinical treatment response and judge the boundary between the normal skin and skin scar. With the advent of the minimized non-linear spectral imaging apparatus, we have enough reasons to believe that the non-linear spectral imaging technique will become a valuable tool to noninvasively study the pathophysiology of human hypertrophic scar and assess its treatment response in clinic or track its development in the laboratory and clinic without fixation, sectioning, and the use of exogenous dyes or stains. And this technique is also further developed to investigate the pathological

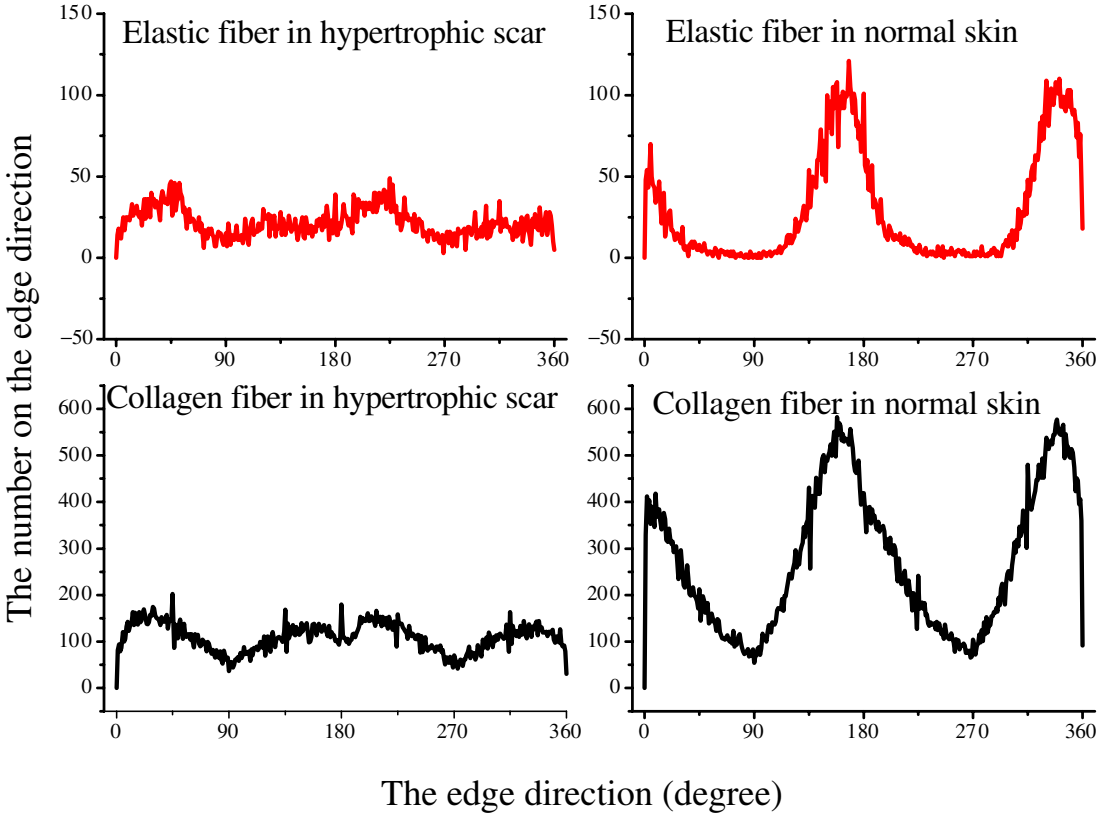


Fig. 3. The edge direction histograms of collagen and elastic fibers in the normal skin and hypertrophic scar tissue.

differences between keloid and hypertrophic scar, which are two common types of excessive scarring observed clinically that require different therapeutic approaches.

Acknowledgments

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