Multiphoton imaging of excised normal skin and keloid scar: preliminary investigations

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ABSTRACT

Wound healing is a physiologic process that acts to repair disruptions in the continuity of tissue caused by injury or surgical incision. Keloids and hypertrophic scars are forms of aberrant wound healing, which are characterized by the overproduction of collagen, resulting in an excessive amount of scar tissue. Keloid tumors, by definition, grow outside the boundary of the original tissue damage. Multiphoton microscopy (MPM) is an imaging technique which allows imaging of living specimens, without the use of fixation or stains. Images of collagen fibers are produced by the second harmonic signal intensity generated by endogenous fluorescence through excitation by infrared laser light. A postauricular keloid tumor was excised from a patient. The tissue was dissected, and a portion was imaged using MPM. Normal skin tissue was isolated from a patient undergoing a facelift. A portion of this tissue was also dissected and imaged using MPM. MPM images were taken using a 63X water immersion objective lens on a two-photon microscope and a titanium-sapphire laser. Images were taken beginning at the surface of the tissue and moving in at intervals of 200 nm to a final depth of 30 μm. The two-photon images were used to reconstruct three-dimensional representations of the collagen matrix within the tissues, which are readily contrasted. Density of the collagen within each tissue was also ascertained using depth dependant decay of the image intensity. Multiphoton imaging was successfully used to image the collagen matrix of normal skin and a keloid scar, demonstrating differences in their microstructures.

Keywords: keloid, multiphoton microscopy

1. INTRODUCTION

1.1 Aberrant healing

Wound healing is a physiologic process employed by many organisms that acts to repair disruptions in the continuity of tissue caused by surgery or trauma. Wound healing consists of hemostasis to prevent excessive blood loss, inflammatory processes to prevent infection, and extracellular matrix deposition and remodeling [1]. These processes usually occur seemlessly, and traumatized tissue is replaced by scar. In some cases, however, aberrant wound healing occurs, producing excessively large scars. These scars may be cosmetically unacceptable and can lead to pain, pruritus, and joint immobility [2]. Keloids and hypertrophic scars are forms of aberrant wound healing, which are characterized by overproduction of collagen, resulting in an excessive amount of scar formation. Hypertrophic scars are characterized by excessive scar tissue production that remains confined by the borders of the original tissue insult. In contrast, keloid tumors are defined as benign neoplasms that grow beyond the boundary of the original region of tissue injury [3, 4]. They are unique to humans, and no animal models have been developed to simulate these tumors [5]. Keloids differ from hypertrophic scars in that they do not regress or contract, and they have a tendency to recur after surgical removal. Keloids are equally distributed between males and females, and are seen in all races, however, they are much more common in darker skin types [1].

1.2 Treatment and study

The current treatment for keloid tumors includes surgical excision followed by an adjunct treatment such as corticosteroid injections. Surgery alone results in a very high rate of recurrence. Cryotherapy has also been used as a form of treatment with some success, and some studies have even shown the benefit of freezing tumors from within using needles [6]. Other therapies include irradiation of the keloid [7, 8], laser treatment, or injection of cytotoxic drugs [9, 10]. At present, however, the treatment of keloids remains more art than science, and clinicians rely more upon on trial and error. Some researchers have suggested that there may be a genetic predisposition to the development of keloids based

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on circulating levels of growth factors including TGF-β [5, 11]. Various techniques have been developed to study keloid tissue both *in vivo* and *in vitro*. Most *in vivo* studies have consisted of small clinical trials with various treatment methods [6, 7, 8, 9], while most *in vitro* studies involve the culture of keloid fibroblasts and/or keratinocytes [12, 13], and the measurement of cell proliferation, collagen formation [14], or cytokine production [2, 15, 16]. Collagen in keloid tissue has already been imaged using both immunohistochemical methods and electron microscopy in previous studies [17]. In this study we add multiphoton microscopy to this list.

1.3 Multiphoton microscopy

Multiphoton excitation fluorescence microscopy (MPM) is an emerging imaging techniques in biology, and has been described as "the best noninvasive means of fluorescence microscopy in tissue explants and living animals" [18]. This imaging method employs many advantages over conventional imaging techniques such as confocal or electron microscopy. Tissue specimens do not require fixation, dehydration, or staining. In fact, MPM can be used on live tissue without causing any adverse effects. Images are produced through the laser-induced excitation of endogenous fluorophores, including nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), riboflavin, and collagen fibers [19].

1.4 Objectives

In this study, we use the technique of multiphoton microscopy to image the collagen within keloid tissue in order to compare the collagen density to a normal tissue control.

2. METHODS

2.1 Attaining Tissue Specimens

Tissue specimens were isolated from patients undergoing surgery at the University of California, Irvine Medical Center. These procedures were all completed under the aegis of UC Irvine Institutional Review Board protocols. Tissue was labeled as either *normal* or *keloid* based on its origin. Normal tissue was obtained from the post-auricular region of an elderly Caucasian woman undergoing a rhytidectomy. Keloid tissue was taken from a keloid tumor removed from the post-auricular crease of a 55-year-old Mexican American male. The patient was of dark complexion and had an additional hypertrophic scar on his nose. The patient's keloid tumor had formed secondary to surgery, in which an incision was made originally to harvest a cartilage graft from the concha of the pinna. The keloid failed to respond to serial steroid injections, and had been excised once before surgically. Immediately following excision, both tissue specimens were placed on ice and transported to Beckman Laser Institute at UC Irvine for processing and imaging. Once in the laboratory, the tissue was serially washed with a PBS solution containing gentamicin and amphotericin B in order to eliminate any contaminating microorganisms. A 1 cm^2 portion of each tissue specimen was then taken for imaging by two-photon microscopy.

2.2 Multiphoton microscopy images

Two-photon images were taken using a titanium-sapphire (Ti-Al_2O_3) laser (Coherent – Santa Clara, CA) and a Zeiss Axiovert S100 2TV microscope (Zeiss – Thornwood, NY) with a 63X water immersion objective lens. A detection system capturing second-harmonic signal produced by the excitation of collagen by infrared laser light was used to produce two-dimensional images [19, 20]. The titanium-sapphire laser was set to emit light of a 780 nm wavelength, and fluorescence was detected by a system with two photomultiplier tubes - one detecting green light (R7400P), the other detecting red light (R7400P-01) (Hamamatsu – Bridgewater, NJ) [21]. Imaging was started at a random central location on the internal dermal surface of each tissue specimen, and additional images were taken at increasing depths into the tissue using a PC-controlled galvanometer-driven X-Y scanner (Cambridge Technology – Watertown, MA). Two-photon images were taken at intervals of 200 nm to a final depth of 30 μm, resulting in a total of 200 images per tissue sample. This stack of two-dimensional images essentially gives a three-dimensional picture of the collagen within the tissue specimen. Images of the fluorescence produced by collagen content were saved for later depth dependant decay analysis.

2.3 Depth dependant decay

Relative collagen density of the tissue samples was measured using depth dependant decay analysis of the two-photon images. Images were analyzed using MATLAB (The Mathworks – Natick, MA), which assigned a numerical value to

the signal intensity for each image. The data were then plotted as intensity versus depth, and exponential trendlines were created to fit the data to the following equation:

$$y = Ae^{-kx} + C \quad (1)$$

where y represents second harmonic signal intensity, x equals the depth from the specimen surface, k is the depth dependant decay constant, A is a pre-exponential scaling factor, and C is a constant which adjusts the lowest signal intensity to zero. Finally, the negative sign denotes an exponential decrease or decay. These calculations were made using Excel (Microsoft – Redmond, WA). The depth dependant decay constant (k) was used to compare the rate of signal decay and thus density of collagen in the tissue samples.

3. RESULTS

Depth dependant decay analysis of the two-photon images provided a quantitative means of comparing the relative collagen density of normal skin versus that of keloid scar tissue. By fitting the data to the exponential trendline provided in equation 1, depth dependant decay constants were obtained, which represent collagen density in the tissue. The depth dependant decay constant (k) for normal tissue was 0.036, while k for keloid tissue was 0.017. This indicates that the collagen density of the normal tissue was 2.1 times that of the keloid tissue. Figure 1 shows the data plots and the trendlines for both normal and keloid data. The trendlines clearly show a steeper decay within the normal tissue compared to the keloid tissue. This signifies a faster decrease in signal intensity with depth for normal tissue than for keloid tissue due to the increased amount of collagen dampening the fluorescence signal. The coefficient of determination or R-squared value for the trendlines are 0.8973 and 0.9607 for normal skin and keloid scar respectively.

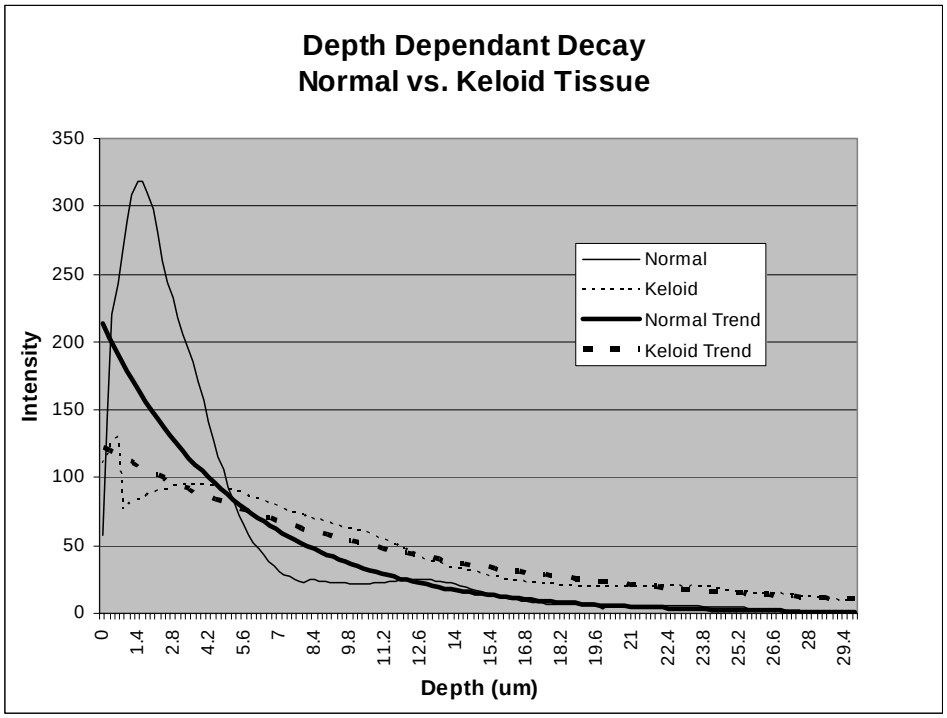

Figure 1. Depth dependant decay curves showing the exponential decrease in signal intensity as depth increases for both normal and keloid tissues. Thin lines represent raw data while thick lines represent trendlines. Solid lines show normal data while broken lines show keloid data.

4. CONCLUSIONS

4.1 Discussion of results

These preliminary investigations of the collagen density of human skin as illustrated by two-photon microscopy have shown normal skin to have a higher collagen density than keloid tissue. As keloid tissue is thought to be made up of dense collagen deposits, this is indeed a surprising and interesting result. While this is merely a preliminary finding and additional studies will be necessary to test the reproducibility of these results, it is useful to propose possibilities for this interesting outcome. A first possibility is that this low density collagen is a unique characteristic of the particular keloid used in this study, or may be an artifact related to the injection of local anesthetics into the tumor (though the facelift tissue was injected with anesthetic solution as well). Keloid tumors show great variation with regards to size, rate of growth, cell content, and response to growth factors [1]. Certainly the density of collagen could vary between keloids as well, and we may have isolated a particularly low-density sample that was also treated many times with intralesional steroid injections. Further studies with additional keloid tissue will determine this. Another possibility is that while keloids form a large amount of collagen, this collagen is arranged in a loose, disorganized manner. It has been shown that keloid tissue has an increased amount of water in comparison to normal tissue [22]. This increased water content and loose collagen arrangement could account for the lower density of collagen in keloid tissue.

4.2 Future directions

Keloids and hypertrophic scars continue to be a problem for both patients and the surgeons and physicians treating them. The definite cause and the treatment for these unaesthetic, painful, and even debilitating [2] lesions remains to be fully elucidated. This study has attempted to add a new form of microscopic visualization of the microstructure of keloid tissue to the continually growing number of imaging methods already employed. The more that is understood about the structure and composition of these scars, the closer we will be to finding a successful treatment for them. Unfortunately, especially for us researchers in Orange County, CA, keloid tissue is not easy to obtain. Patients with keloids are rare at the UCI Medical Center, so we must use each sample to its fullest potential. We continue to look for new sources of keloid tissue and new ways to investigate the characteristics and behavior of these scars.

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