In vivo detection of cryosurgery using multiphoton and harmonic generation microscopy

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Abstract

The tissue injury and the organization of collagen during cryosurgery are poorly characterized because of the lack of appropriate methodologies. In this study, we use multimodal multiphoton microscopy to assess the change of extracellular matrix after cryotreatment of skin. The cellular matrix transformations and the intercellular interactions during the wound healing process after cryolesion for mice were measured *in vivo* and in real-time through the dorsal skinfold chamber (DSC). Intrinsic second-harmonic generation (SHG) signals from fibrillar collagen and two-photon excited (TPE) autofluorescence from cell were collected to investigate the cryosurgical response *in vivo*. The TPE and SHG signals are significantly different between normal and cryotreated mice, and correlates with the wound healing process. The results suggest that this approach may be applied in real-time to noninvasively monitor the cryosurgery process and could potentially be applied to clinical evaluation. © 2008 IPPEM. Published by Elsevier Ltd. All rights reserved.

Keywords: Second harmonic generation (SHG); Dorsal skinfold chamber (DSC); Two-photon excited fluorescence (TPE); Cryosurgery

1. Introduction

Cryosurgery is a process of freezing tissue to produce a local destructive effect. In order to provide fast cooling to destroy cells and tissues, cryogens such as liquid nitrogen, liquid argon, or carbon dioxide were used. Cryosurgery is low cost, safe and relatively simple to perform. It is also a minimally invasive treatment for prostate, liver, breast and skin cancer. This technique is also an excellent choice of treatment for many skin diseases including actinic keratosis, melanoma, basal and squamous cell carcinomas [1,2].

Understanding the injury mechanisms in cell, tissue and organ is important for the development of new and efficient cryosurgical techniques. There are three major hypotheses describing the mechanism of tissue injury in cryosurgery: cellular injury, vascular injury and freezing-stimulated immunologic injury [3]. Cellular injury is the irreversible tissue damage from intracellular ice formation and protein damage from high solute concentrations [4]. Vascular injury is due to the microcirculatory stasis and local ischemia in tissue. Freezing-stimulated immunologic

injury results from the cryoimmunological effect activated by cryosurgery [5–8]. Although one is not necessarily more important than the other, this study will focus on the cellular injury hypothesis by monitoring the cellular matrix transformations and intercellular interactions during the wound healing process after cryosurgery.

Conventionally, the approach to study or to monitor cellular injury is through histological fixation and labeling procedures. Because of the invasive nature of histological treatments, studies on the temporal process of wound healing of frosted tissue are rare. For many years, the dorsal skinfold chamber (DSC) has been used for intravital microscopy observation in *in vivo* animal model [9–11]. The animals tolerated DSC very well and exhibited the same habitual behavior. Since the nude mouse skin is shown to be similar to the human skin [12], nude mouse mounted with DSC can be a good animal model for monitoring wound healing of cryolesions using nonlinear optical microscopy.

Multiphoton and harmonic generation microscopy is a novel technology for nondestructive analysis of tissue. These nonlinear optical processes involve point-like excitation which reduces photo-damage and photo-bleaching [13]. Furthermore, both processes have the advantage of not requiring any staining. Images are generated from both laser-excited

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autofluorescence and second-harmonic signals. The second-harmonic generation (SHG) technique is a powerful tool and has been employed to study and obtain images of biological materials [14–16]. Specifically, SHG imaging is well suited to study the bulk of noncentrosymmetric materials, such as collagen fibrils [17,18]. Since type I collagen is the most abundant protein in the body, changes in skin collagen structure caused by cryosurgery could be studied using SHG.

The two-photon excited fluorescence (TPE) offers several advantages for monitoring living cells or tissues [19]. In recent years, researchers have widely reported on *in vitro* or *in vivo* high-resolution multiphoton imaging of skin [20] or cornea [21]. TPE could detect fiber structures such as elastin which has an emission wavelength around 405 nm [22]. Furthermore, the TPE autofluorescence intensity could be used as an index factor for the cellular redox metabolism because the TPE signal is consistent with molecules such as NAD(P)H, flavoproteins and tryptophan [23].

This study presented an *in vivo* and real-time approach for investigating the tissue injury during a typical cryosurgical process. By using the DSC with the combination of TPE and SHG microscopy, the wound healing process after cryosurgery was studied in a noninvasive and real-time manner.

2. Materials and method

A total of 12 nude mice (BALB/c-nu, National Laboratory Animal Center, Taiwan) were used in animal experiment.

Fig. 1. Photograph of a mouse mounted with DSC.

The mice were 5–6 weeks old and with a body weight of 20–22 g. Experiments were conducted according to the guidance of the animal protection law for animal experiments. The animals were housed one per cage and had free access to water and food throughout the experiment. DSC was surgically mounted onto each mouse. The DSC shown in [Fig. 1](#) consists of two frames with a cylinder-shaped push piece located on the opposite side of the frame. DSC was made of medical-graded titanium alloy and allowed measurement and observation of the experimental animals for 10 days. After surgically mounted the DSC, the animals were allowed to recover from anesthesia and surgery for at least 2 days. After cryosurgical lesion, the tissue responses were observed using TPE and SHG microscopy. Dorsal skin images of normal and

Fig. 2. Schematic diagram of the TPE and SHG imaging experimental setup.

Fig. 3. SHG intensity of porcine skin collagen from -196 to 50 °C.

Fig. 4. AF intensity of porcine skin collagen from -196 to 50 °C.

cryotreated mice were obtained on Days 0, 1, 2 and 4 from the chamber window at various depths.

A schematic diagram of the two-photon microscope used in this study is shown in Fig. 2. The image system was modified from an upright microscope (E800, Nikon, Japan). A 780 nm titanium-sapphire laser with 100 fs pulse (Tsunami, Spectra Physics) is the light source for the imaging system. Intrinsic autofluorescence was gathered in the epi-fluorescence mode through a filter (435–700 nm, E435

LP 700 SP, Chroma Technology) and onto a photo-multiple tube (PMT) detector (R7400P, Hamamatsu City, Japan). The SHG signals of collagen were collected using a second PMT detector through a different filter (HQ 380/40, Chroma Technology). A high-resolution imaging, high numerical aperture, oil-immersion objective (S Fluor 40X, NA 1.3, Nikon) is used for both TPE and SHG microscopy. The Image J program was utilized to analyze and process the SHG and TPE data.

Fig. 5. Images of normal and cryotreated mice taken at depths of 0, 40, 80 and 120 μm. (a–d) Normal mice, (e–h) cryotreated mice (SHG, blue; TPE autofluorescence, green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Results and discussion

Figs. 3 and 4 shows the SHG and TPE autofluorescence (AF) intensity of skin collagen under the temperature treatments of -196 , -80 , -20 , 0 , 4 , 25 , 37 , 40 , 45 and 50 °C. The SHG intensity decreased significantly for the treatments below 20 °C or above 25 °C. This suggests a correlation between tissue damage and decreased SHG intensity. The AF intensity displays similar result to the SHG intensity, and also suggested a relationship between tissue damage and decreased AF intensity.

For the lower temperature treatments (-196 , -80 , -20 °C) and higher temperature treatments (37 , 40 , 45 , 50 °C), the SHG intensity was about half of the SHG value in the moderate temperature region from 0 to 25 °C. Above 25 °C, the thermal effect can result in structural instability of the collagen. As the collagen structures were disrupted by the thermal effect, the SHG intensity decreased at higher temperature. In addition, fibrillar collagen could lose its fiber structure and reduced the SHG signals. Similar reduction of the SHG intensity was observed in the lower temperature range. These results could be attributed to the formation of ice crystals, which might disrupt the structure of collagen fibrils and lower the SHG intensity. Since the TPE and SHG signals of collagen decreased at low temperature, they could be used to monitor tissue damage and wound healing.

TPE and SHG microscopy images of normal and cryotreated mouse skin tissue at various skin depths are shown in Fig. 5. From the series of images, structures such as stratum corneum, stratum granulosum, stratum spinosum and stratum basale could be identified.

Both Fig. 5a and e shows green autofluorescence of the stratum corneum with polygonal corneocytes at the skin surface. At the skin depth of 40 μm for normal mice in Fig. 5b, there were strong TPE signals from the spinous cells of the stratum spinosum. These spinous cells shaped like donut with their cellular cytoplasm showing brighter fluorescence than the nucleus. However, the spinous cells were not observed at the depth of 40 μm for the cryotreated mice (Fig. 5f). Similarly, the basal cells of the stratum basale were also observed in normal mice, but was absent in cryotreated mice. The cell morphology discrepancy indicated the difference between the living cells in normal mice and the dead cells in cryotreated mice.

At a depth of 80 and 160 μm , blue SHG signals from the collagen and the green TPE signals from the elastin were both observed in Fig. 5c, d, g and h. Furthermore, elastin fiber structure entangled with collagen structure to form networks in the dermis. For the cryotreated mice in Fig. 5g and h, there were lesions containing necrotic cells that caused a decrease in the TPE signal. Fibroblasts, indicated by the red arrows, could also be identified in the image. There were also an absence of epithelial cells, which might be caused by the irreversible tissue damage resulted from the intracellular ice formation.

Fig. 6. (a) SHG intensity profiles over 4 days and (b) TPE autofluorescence intensity profiles over 4 days.

After measuring the normal TPE and SHG signals before cryotreatments on Day 0, Fig. 6 shows the intensity profile of mice skin over a period of 4 more days after cryotreatment. Fig. 6a suggests that the SHG intensity showed a general trend of increase from the first day to the fourth day, but did not recover to the pre-treatment level in Day 0.

Fig. 6b shows that the AF intensity decreased at Day 1 after the cryotreatment, but recovered to the pre-treatment level on Day 2. The decreased signal from Day 1 was due to the epidermal cell damage caused by the extreme temperature of liquid nitrogen. On Day 4, the autofluorescence increased to three times the initial value, which could be attributed to the proliferation of stratum corneum in the lesion tissue.

Analysis of tissue damage after cryotreatment in the DSC revealed an excellent correlation between the TPE intensity of cell and the SHG signal of collagen for Days 1, 2 and 4. The TPE and SHG intensities correlated with the cellular damage resulted from extreme temperature treatment of liquid nitrogen. Therefore, this method is helpful for better understanding and administrating the operational planning of the cryosurgical process. The ability to detect the cryosurgical changes noninvasively in real-time could potentially be applied to clinical evaluation.

4. Conclusion

The wound healing process of cryosurgery was monitored by the TPE and SHG microscopy in mice mounted with DSC. The TPE and SHG signals of fibrillar collagen were shown to decrease at low temperature, and could be used as an index factor for wound healing. The TPE and SHG images between normal and cryosurgery mice showed significant differences which could be attributed to the tissue damage from the cryotreatment. The TPE and SHG signals also correlated with the wound recovery process. These results suggested that the TPE and SHG microscopy combined with DSC could be a valuable animal model to provide information on the wound healing processes at the cellular level after cryosurgery or injury. The ability to detect these changes noninvasively in real-time could potentially be applied to clinical evaluation to improve the understanding and the operation planning of the cryosurgical process.

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Conflict of interest

There is no conflict of interest with employment, financial benefit, personal relationships, professional relationships or other interests in preparing this manuscript.

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