A low fluence Q-switched Nd:YAG laser modifies the 3D structure of melanocyte and ultrastructure of melanosome by subcellular-selective photothermolysis

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Abstract Laser treatment using low fluence for melasma was previously introduced to overcome postinflammatory hypermelanosis after Q-switched laser therapy. However, research on the mechanism of this treatment is very limited. In this study, a collimated low fluence 1064 nm Q-switched Nd:YAG laser with a pulse width of <7 ns was applied using top-hat beam mode. The aim of this study was to investigate the mode of action of this laser treatment through electron microscopy. The effectiveness of this treatment was confirmed by clinical photos, melasma area and severity index and spectrophotometer. To understand the mode of action, the three-dimensional structure of melanocytes in the epidermis was analyzed using serial images acquired by a 3VIEW surface block face scanning electron microscope. In the epidermis, after laser treatment, fewer dendrites in the melanocytes were observed compared with pretreatment. In addition, ultrastructural changes in the melanosome were studied using transmission electron microscopy, which showed that laser treatment caused selective photothermolysis on Stage IV melanosome. Therefore, this treatment should be regarded as an effective method for treating melasma through subcellular-selective photothermolysis.

Keywords 3VIEW SBF-SEM, 3D structure, melasma, melanocyte, melanosome, laser treatment

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Introduction Melasma is a commonly acquired hyperpigmentation and a common dermatologic skin disease that occurs on sun-exposed areas of the skin [1,2]. The methods of treatment include melanin biogenesis pathway blockers, depigmenting agents, chemical peels, dermabrasion and laser treatments [3]. The topical agents including hydroquinone and tretinoin are the most well known treatment for melasma [3,4], but the treatments have not been very effective for Asian skin types. The main reason is that these agents have limitations in removing the persistent overproduced melanin within the epidermis and dermis. Therefore, the importance of approaches that physically remove melanin or destroy melanosomes such as laser treatment is being magnified [5,6]. So far, laser treatments have been attempted with few successes and many
failures, which result from induced postinflammatory hypermelanosisis [5,7–14]. These failures were caused by the lysis of cells that contain melanosomes due to heating from the high fluence of the laser [9]. Therefore, treatments using low fluence lasers were introduced [3]. However, no clinical studies with randomized controlled trials have been reported and the mechanism by which this method removes melanin-containing melanosomes is still not known. In this study, we examined the structural modifications of melanocytes and melanosomes after exposure to a Q-switched Nd:YAG laser using low fluence for the minimal thermal damage.

Melanocytes are cells located in the bottom layer of the epidermis and their dendrites are scattered in all layers of the epidermis [15,16]. The dendrites of melanocyte are too small to be resolved by three-dimensional (3D) light microscopy techniques such as confocal or two-photon microscopy. Only electron microscopy has sufficient resolution to image them. Traditionally, 3D reconstruction of these structures has been achieved by serial section transmission electron microscopy (SSTEM) using ultrathin sections [17]. However, the reconstruction of large volumes using SSTEM has been limited by the daunting task of serial sectioning and reconstructing thousands of sections. Recent technological developments have improved upon the disadvantages of SSTEM through automated section and acquisition [7,18]. In this study, we investigated the mode of action of the Q-Switched Nd:YAG laser through electron microscopy by employing 3VIEW surface block face-scanning electron microscopy (SBF-SEM), and transmission electron microscopy. The 3D structure of melanocyte with adequate resolution to identify dendrite connections was reconstructed using serial images acquired by 3VIEW SBF–SEM. Ultrastructural changes of melanosomes in melanocytes after laser treatment were examined using transmission electron microscopy.

Materials and methods

Laser treatment
Sixteen subjects having symmetrical distribution for clinical diagnosis of melasma were enrolled in the study. An explanation including the risks, benefits and potential complications was provided to the patients, and written informed consent was obtained. All subjects were assigned to the split-face study, and treated with 5–7 ns pulse width, 1064 nm wavelength and 7 mm spot size using Q-switched Nd:YAG laser (Spectra VRMIII®, Lutronic Corp., Seoul, South Korea). The fluence was lowered to 1.6–2.0 J and the treatments were repeated only two times regardless of the development of erythema. The treatment was repeated weekly for 8 weeks, and after that topical steroid ointment (Dermatop®, prednicarbate 0.1%) was followed for the reduction of inflammation.

Assessment of response
The effectiveness of this treatment was evaluated by clinical photos, melasma area and severity index (MASI) scoring system, spectrophotometer (Minolta CM-2006d®, Japan) measurements and patients’ subjective evaluation. Clinical images were photographed by an evaluator using a digital camera (Sony DSC-F828®, Japan) at each treatment session. The treated side was evaluated and compared with the opposite nontreated side using MASI scores. In addition, a spectrophotometer was used to measure skin color and brightness based on three measurement values ($L^*$, $a^*$, $b^*$) and $\Delta E^*ab$ value. Sampled areas both with and without laser treatments were read by the spectrophotometer in triplicate, and the values were averaged.

Sample preparation for ultrastructural study
To investigate the ultrastructural changes immediately after laser treatment, biopsies were obtained from subjects both without and with treatment. Biopsies were taken from the least conspicuous site within the clinically relevant area in five patients. Samples were immediately fixed with a 2.5% glutaraldehyde-mixed 2% paraformaldehyde solution (0.1 M cacodylate buffer, pH 7.0) for 2 h, and then followed by postfixation with 2% osmium tetroxide for 2 h at 4°C. The block was stained in 2% uranyl acetate and dehydrated with a graded acetone series. The samples were then embedded into Spurr medium (EMS, USA).
3VIEW – serial imaging in SEM
The block face was trimmed using either a conventional microtome or a sharp knife. SEM images of the untrimmed block face were used to select the desired field of view before the final trimming step, producing the desired small cut pyramid. Five hundred serial images were taken using an environmental SEM Quanta FEG 200 (FEI, Netherlands) with a 3VIEW serial block face sectioning and imaging system (Gatan, USA) at an accelerating voltage of 5 keV. The equipped diamond knife was custom made by diatome (Diatome, Switzerland), and the section thickness was 50 nm. Images were collected at imaging size of 1 k × 1 k, pixel size of 50 nm, pixel dwell time of 80 μs, chamber pressure of 0.50 Torr and spot size of 3.

Transmission electron microscopy
Samples were sectioned with 60 nm thickness using an ultra-microtome (RMC MTXL, USA), and double-stained with 2% uranyl acetate for 20 min and Reynolds lead citrate for 10 min. The sections were then viewed under a JEM-1400 (JEOL, Japan) at 80 keV. The images of TEM were taken using 4008 × 2672 pixel digital camera (CCD MORADA, Olympus, Germany) at 10K and 40K magnification.

3D structure reconstruction
IMOD software (Colorado Univ, USA) was used for 3D rendering of serial images using SBF–SEM.

Results
A low fluence toning treatment of Q-switched Nd: YAG laser improved melasma
All of the 16 patients in the study completed treatments without any adverse reaction. To prevent overtreatment, the fluence was lowered to 1.6–2.0 J and treatments were repeated only two times. Their MASI score was lowered from 5.14 ± 4.62 to 1.84 ± 2.20, which was a statistically significant change (P < 0.01). Spectrophotometer measurements were performed using a Minolta CM-2006d®. The L* value improved from 58.83 ± 4.14 (pretreatment) to 60.62 ± 3.87 (posttreatment) and the ΔE*ab value improved from 6.29 ± 3.20 (pretreatment) to 3.75 ± 2.07 (posttreatment) (P < 0.001; Table 1).

Electron microscopic serial images and 3D structure using serial images reveal reduction of melanosome and melanocyte dendrites in the melasma epidermal layer after Q-switched Nd: YAG laser treatment
For electron microscope observation, resin blocks of the biopsy from melasma patients without and with laser treatment were made. Through serial photography of 500 sheets with 50 nm thickness using 3VIEW SBF–SEM, the structure of melanocytes were analyzed to a depth of 25 μm in the epidermis layers without and with treatment. Figure 1a and b shows each one of these serial image sets. Because a backscattered electron detector was not available, the images were produced using a secondary electron detector, which is less informative than a backscattered electron detector.

Table 1. Evaluation of the effectiveness of laser treatment

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex/age</th>
<th>Pattern</th>
<th>Pretreatment</th>
<th>Posttreatment</th>
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<td>L* value</td>
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<td>Malar</td>
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<td>3.05</td>
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used for imaging, each image has a similar contrast compared with that using transmission electron microscopy (Fig. 1a and b). Melanocytes were represented as contour of dark green, and melanosomes were showed in Fig. 1c and d through colored spots. Through these images, the reduced volume of melanocytes and decreased number of melanosomes were observed in the epidermis with laser treatment (Fig. 1c and d). However, it was difficult to discriminate connecting dendrites from the 2D images. Therefore, the 3D structure was reconstructed from these serial images, and the structural changes of dendrites in melanocytes could be compared through a surface-rendering model using the IMOD software. Figure 2a and b shows 3D volumes of 500 and 650 serial images using the 3VIEW SBF-SEM system. They are aligned serial images having dimensions of each $28 \times 28 \times 25 \, \mu m$ (Fig. 2a) and $28 \times 28 \times 32 \, \mu m$ (Fig. 2b) without optical distortion. The 3D structure was reconstructed using the 500 serial sections. Each image was combined using the IMOD program and the outline of the melanocytes’ body and their dendrite was extracted in the contour form. These contours were connected and reorganized to generate a topographical map of the melanocyte. Figure 2c and d shows one of the serial images including reconstructed melanocytes as 3D structures. As a result, it was showed that
dendrites of melanocyte in melasma patients were distributed from the basal layer to the granulosum layer (Fig. 2e). In contrast, we found that the dendrites of melanocytes barely stretched out any further after laser treatment (Fig. 2f).

Transmission electron microscopy showed the difference of melanosome in melanocyte with and without laser treatment

Serial images of SBF–SEM showed a decrease of Stage IV mature melanosomes. To investigate the
ultrastructure of melanosome, we observed the melanosome in melanocyte using transmission electron microscopy. As a result, Fig. 3 shows that melanosomes are in rod shape and compact in patient without treatment (Fig. 3a, c and e). In patients after laser toning treatment, remnants of

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**Fig. 3.** Transmission electron microscopy images of melanosomes in melanocytes of melasma patient. The image of melanocyte from a melasma patient (a, c and e) and that after postlaser treatment (b, d and f) were compared for investigation of the ultrastructural change of melanosome after laser treatment. Melanocytes in melasma patients without laser treatment have many melanosomes (a, c and e). After laser toning treatment, the melanocytes had far fewer melanosomes (b, d and f) and remnant (d) and lysed form (f) of Stage IV melanosome in melanocyte was observed. Arrow of (d) shows the remnant of Stage IV melanosome. Lysed Stage IV melanosome was represented as circle in (f). However, melanosomes in the early stage like Stages I and II were observed without any change (d and f). The size bar of (a) and (b) is 2 μm and that of (c–f) is 0.2 μm.
melanosomes were observed in the images of melanocytes (Fig. 3d). The remaining melanosomes had a lower electron density and appeared to exist in the lysed form (Fig. 3f). However, no structural changes were observed in Stage I and II melanosomes after laser treatment (Fig. 3d).

**Discussion**

There are many treatment modalities for melasma; however, there is no sure-fire method of treating this disease. In the past, laser treatment has failed because of the resulting inflammation, consequent pigmentation and excessive thermal damage caused by the use of high fluence [9]. Recently, lasers with low energy and low density were suggested as a potential promising alternative method to treat melasma [19,20]. Among these lasers, the pulsed radiation of the Q-switched Nd:YAG laser has been shown to remove pigmentation by selectively destructing pigment cells with a short pulse and low fluence. However, the mechanism by which the laser treatment removes melanin within melanosome is still not known.

To investigate the mode of action of this laser treatment, we analyzed the structural changes of melanocytes using 3D electron microscopy. Among 3D electron microscopy techniques, 3VIEW SBF–SEM offered important advantages compared with the traditional SSTEM technique [21]. First of all, as the face of the resin block was directly photographed, the problem of section distortion or loss while handling was solved. In addition, as the images of sections were automatically aligned with no need for manual alignment, reconstruction of 3D structures was easy. All of the over 500 serial images confirmed that there was a decrease of melanosomes after laser treatment. Figure 1 shows one section among over 500 sections. This phenomenon was also reported in previous studies that used Q-switched ruby laser irradiation [22] or erbium and glass laser treatment in the fractional mode [8].

In addition, Fig. 2 shows that the volume of the melanocytes in melasma patients is reduced significantly and the change of dendrites is observed in the epidermis with laser treatment. The 3D structure of melanocytes showed that dendrites were stretched from the cell body located at the basal layer to the granulosum layer of the epidermis in melasma patients without laser treatment. However, we observed a decrease in the number of connecting melanocyte dendrites after laser treatment through reorganization of the 3D structure of melanocytes. Pearl et al. reported that the number of melanocytic dendrites in melasma patients was increased compared with normal skin [13]. The decrease in melanocytic dendrites after laser treatment may have occurred because of the thermolytic effect of the laser on melasma. Because mature Stage IV melanosomes are accumulated in the dendrites of melanocyte, the selective photothermolysis effect of the laser can be focused intensively in dendrites [23–25].

Ultrastructural changes of the melanosomes in the melanocytes with laser treatment were compared with that without treatment by the transmission electron microscopy. In this study, images from transmission electron microscopy revealed alterations of mature melanosomes in melanocytes (Fig. 3). Therefore, this result can also be interpreted as selective photothermolysis effect of the laser on mature melanosome.

In summary, by examining the 3D structure through SBF–EM and the image from transmission electron microscopy, we found that treatment of human melasma skin with a Q-switched Nd:YAG laser resulted in the decreased number of melanocytic dendrites and altered ultrastructure of melanosome. These results revealed that this laser treatment lysed the subcellular-specific organelle, melanin. Therefore, we suggest the collimated low fluence Q-switched Nd:YAG laser is an effective method for the control of melasma through low energy resulted in subcellular-selective photothermolysis (SSP).

**Conclusions**

Our results show the mode of action of laser treatment for melasma referred to as ‘laser toning’ through electron microscopy. In detail, the clinical effect of Q-switched Nd:YAG laser with short pulse width and low energy demonstrated good therapeutic results. We revealed that its mechanism of action is SSP by 3D structure using serial block-face SEM. In addition, we also confirmed SSP of this laser treatment through ultrastructural change of melanosome employing transmission electron microscopy.
Therefore, we can conclude that collimated Q-switched Nd:YAG laser is an effective method for therapy of melasma and the mode of action is SSP.

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