Research Article

Raman Spectroscopic Analysis of H₂O₂-Stimulated Three-Dimensional Human Skin Models Containing Asian, Black, and Caucasian Melanocytes

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Received 11 September 2012; Accepted 17 November 2012

Academic Editor: Kong-Thon Tsen

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Reactive oxygen species generated in dermal cells of human skin is related to skin disorders or diseases. In this study, Raman analysis effectively clarified the identities of three types of human skin models after the models were stimulated with hydrogen peroxide. With the Caucasian skin model, the major Raman bands underwent large intensity changes within 4-5 days of stimulation. With the Black skin model, the Raman bands remained almost unchanged. The changes in the Asian skin model were unique compared to those in the above two. Eumelanin and pheomelanin are probably the main compounds that differentiate dermal cells in terms of their sensitivity to hydrogen peroxide.

1. Introduction

Human skin is sensitive to sunlight, especially to ultraviolet (UV) light, which generates reactive oxygen species (ROS) in dermal cells. This phenomenon is related to disorders or diseases such as stains, freckles, and cancers of the skin. Many researchers have sought to understand these disorders at the molecular level and are also interested in classifying them [1–3]. In clinical medicine, Raman spectroscopy attracts researchers as a microscopic, nondestructive, and nonlabeling research tool. Raman spectroscopy provides information about the compositions, structures, and interactions of molecules [4], and it has been used as a powerful analytical method in chemistry for 80 years. Its application to clinical medicine has been somewhat slower, beginning during the 1970s [5–7], when the development of a Raman microscope allowed effective spectral measurements of biological tissues [8–11]. Today, we can measure the Raman spectrum of a live cell (ca. 20 μm in width) with high spectral quality, distinguishing an area of 1 μm^2 in the horizontal plane (ca.

30 s for the spectrum) [12, 13]. Moreover, the use of a flexible fiber is one of the most promising approaches for making noninvasive Raman measurements of living tissues, and several research groups have developed fiber optics suitable for Raman measurements [14–19].

In this study, three-dimensional (3D) human skin models were stimulated with hydrogen peroxide (H₂O₂) to provide time-dependent Raman spectra. As mentioned above, UV-visible light generates ROS in dermal cells. H₂O₂, one of typical ROS, was therefore used as a substitute for UV light to stimulate the skin models. The 3D human skin models consisted of keratinocytes, melanocytes, and a collagen-layered membrane, as shown in Figure 1(a), which are frequently used for skin irritation tests in dermatology. Three kinds of skin models, containing Caucasian, Asian, and Black type melanocytes, were used to simulate individual differences. Caucasian-type keratinocytes were used throughout the models.

The aims of this study were (i) to confirm whether the Raman technique is capable of detecting early chemical

FIGURE 1: (a) An example of 3D human skin models containing melanocytes (the hematoxylin and eosin (HE)-stained tissue image). SC, stratum corneum; BL, basal layer; SL, supporting layer; M, melanin. At the right side, a magnified schematic representation around the BL. KC, keratinocyte; MC, melanocyte. (b) The Raman spectroscopic system. The rectangle represents the six-well-plate used to hold the cells; the open circle represents a piece of the skin model; the asterisk indicates the operation of picking a piece of the model skin out of the incubator. The label “(0)” on the vertical axis indicates when the H_2O_2 stimulations were applied to the three types of skin models.

responses or the phenylalanine ring, CH_2 bending, and amide I vibrations within a few days of H_2O_2 stimulation, in the skin models (because pigmentation is recognizable macroscopically only at around two weeks after stimulation) and (ii) to determine whether the Raman approach can clarify/distinguish the identities of these skin models. Our Raman approach successfully clarified the skin properties within 4-5 days after the H_2O_2 stimulation. Not only Raman measurements, we observed also the hematoxylin and eosin (HE)-stained visible images of skin models before and after the H_2O_2 stimulation and the absorbance values using the 405 nm visible light, to give a reasonable discussion. The results obtained in this paper are of importance since they will provide a bright future for a Raman method, mainly utilizing Raman bands of proteins, to enable the nonlabeling, noninvasive, and early diagnosis of human skin disorders.

2. Experimental

Three kinds of 3D human skin models (MEL300 series) and growth medium (EPI-100 containing KGF113) were purchased from the Bio-Medical Department of Kurabo Industries Ltd. from MatTek Corp. Hydrogen peroxide (Wako Pure Chemical Industries, Inc.) was used to stimulate the skin models. A CO_2 incubator was used to maintain the skin

models at 37°C under a 5% CO_2 atmosphere. Five pieces of the Asian skin model were subjected to H_2O_2 stimulation in which $10 \mu\text{L}$ of 2 mM H_2O_2 was added to the 5 mL of medium containing each piece of the Asian skin model. Another piece was used as the control, with no H_2O_2 stimulation. This procedure was repeated for both the Black and Caucasian skin models. Stimulation was applied once for each piece of a skin model.

A CCD-equipped Raman spectrometer (RS in Figure 1(b)) was used with a microscope attachment. Laser light tuned to 785 nm (L785) was reflected with an edge filter (EF). The laser power was averaged to 130 mW. The light was focused onto the target material with an objective lens (L1: 20 $\times$, NA 0.4). The scattered light of the target material reached the RS through L1, EF, a notch filter (NF), an imaging lens (L2), and a slit (S: $100 \mu\text{m}$ in width). For sufficient filtration of the light, the NF filter was placed perpendicular to the path. To establish the time course of the stimulation, as shown in Figure 1(c), the skin model samples were taken out of the incubator piece by piece to make the Raman measurements. We obtained Raman spectra at seven random locations, focusing on the surface of each skin sample avoiding apparent pigmentation spots. The focal point was chosen so that the Raman signal was maximum. The time for the measurement of a spectrum was 600 s (exposure 10 s, with 60 scans).

FIGURE 2: The skin models containing Black type melanocytes (a) without and with the stimulation of the hydrogen peroxide (the hematoxylin and eosin (HE)-stained tissue images). A single piece of the Black type skin model was stimulated by 10 μ L of H₂O₂ (10 mM). (b) The corresponding absorbance values at 405 nm. The error bars correspond to the standard deviation values. The thicknesses of the skin models are approximately from 250 to 300 μ m.

The Raman spectrum, which we called in this paper the “pure” spectrum, was calculated as $y(\nu) = [y_{\text{exp}}(\nu) - a(\nu)]/b(\nu)$, where $y_{\text{exp}}(\nu)$, $a(\nu)$, and $b(\nu)$ are the spectra obtained experimentally with the following procedures. (i) The spectrum $y_{\text{exp}}(\nu)$ was obtained by irradiating the target material on an aluminum foil support with 785 nm light. (ii) The reference spectrum $a(\nu)$ was measured by irradiating the aluminum foil with 785 nm light with no target material (the aluminum foil was placed above the focal point of the light). (iii) Another reference spectrum, $b(\nu)$, was obtained without laser irradiation by placing a white light source at the focal point. A tungsten lump was used as the white light source, and a pin hole was used so that the white light passing through it became a spot (0.9 mm in diameter). The attachment of a variable resistor connected to a battery allowed the light intensity of the light source to be adjusted.

The baselines of the pure Raman spectra were then corrected. The baseline-corrected Raman spectrum $y'(\nu)$ was calculated as $y'(\nu) = y(\nu) - y_{\text{poly}}(\nu)$, in which $y_{\text{poly}}(\nu)$ is a fitted polynomial curve constructed with the following procedures. (i) For a spectrum truncated between the minimum Raman shift position ν_{min} and the maximum position ν_{max} , the degree of the function d was selected to fit the baseline using a polynomial function (this time $d = 8$). (ii) Using the least squares method, the polynomial function y_{poly} was first fitted to the Raman spectrum y . (iii) The Raman spectrum y was divided into upper and lower parts, relative to the fitted baseline y_{poly} . (iv) The number of data points on the upper side of y was designated N_A , and the number on the lower side of y was designated N_B . If $N_A < N_B$, the upper part of y was removed from the whole of y , and the Raman spectrum y was replaced with the lower part of the spectrum.

Then, procedure (ii) was repeated. When $N_A \geq N_B$, the baseline was considered the best fit and optimal.

3. Results and Discussion

As explained in Section 1, human skin is sensitive to sunlight, especially to UV light, which generates ROS in dermal cells. In this research, one of ROS, hydrogen peroxide, was used as a substitute for UV light. Here, we confirmed that the pigmentation of the Black type skin model was reproduced by the stimulation of H₂O₂. Figure 2(a) shows the skin models containing Black type melanocytes without and with the stimulation of the H₂O₂ (10 μ L of 10 mM H₂O₂ was added to the 5 mL of medium containing a single piece of the Black type skin model. The black type was here chosen to exemplify the pigmentation of the skin models since the existence of melanin was the most readily recognizable). The image of the skin model at a week after the stimulation gave several clear spots (almost Black in color) of melanin above the supporting layer (collagen) while that without the stimulation (the control) provided less spots of melanin. Also, we checked that the absorbance value at 405 nm of the skin model after the H₂O₂ stimulation was clearly increased compared to that of the control (Figure 2(b)).

Figure 3(a) shows all the pure Raman spectra produced in the present study. The offset values of the spectra differed greatly. When the background value of a spectrum was low, the corresponding baseline was close to a straight line. If a baseline shifted upward, it was varied to a more concave-down structure, and accompanying this change, the Raman peaks became more planar. In Figure 3(b), the spectra of each model were averaged over the time course. The offset

FIGURE 3: (a) The complete Raman data used in the present research (126 spectra). (b) Time-averaged spectra for the Black, Asian, and Caucasian skin types.

FIGURE 4: (a) Raman spectra after baseline corrections for the Black, Asian, and Caucasian models, respectively. (b) Time-dependent intensity profiles for the Black, Asian, and Caucasian skin types. For the data points, solid circles, asterisks, and open circles are used to represent the intensity values at 1007, 1662, and 1455 cm^{-1} , respectively.

values, wavenumber averaged, were ~ 0.24 , ~ 0.16 , and ~ 0.11 for the Black, Asian, and Caucasian models, respectively. These values are probably influenced by the eumelanin biopolymers of human skin, which gives two broad peaks of Raman bands at 1580 and 1380 cm^{-1} whose full widths of

half heights are around 100 cm^{-1} and whose positions are not influenced by the wavelengths of excitation lights (457.9, 514.5, 632.8, and 785 nm) [20].

Figure 4(a) shows the baseline-corrected spectra. The major bands at 1007 cm^{-1} , 1455 cm^{-1} , and 1662 cm^{-1} are

assigned to the ring breath mode of phenylalanine, the CH_2 bending mode, and the amide I mode of proteins, respectively [13, 21]. Figure 4(b) shows the time-dependent intensity profiles for these specific bands. For the Caucasian model, almost all the Raman bands underwent large intensity changes within 4–5 days of stimulation, and the bands became stable thereafter. For the Black model, the Raman bands remained almost unchanged throughout the observation period. For the Asian model, the spectral changes were intermediate compared with those of the Caucasian and Black models. The Raman responses of the Caucasian and Asian models that occurred within 4–5 days of H_2O_2 stimulation (Figure 4(b)) are both interesting and important because the phenomenon is probably relevant to the early-stage-sensing and diagnosis of pigmentation disorders of the skin (macroscopically, pigmentation was recognized only at 10–14 days after stimulation).

For clarifying whether the spectral changes observed in the above (Figure 4(b)) are specific for effects of reactive oxygen species, the time-dependent intensity changes of the remarkable Raman signals for the Caucasian skin type after the H_2O_2 stimulation (Figure 4(b)) were compared to those without the H_2O_2 stimulation (the control). We confirmed that the drastic changes in the Raman signals within 4–5 days for the stimulated Caucasian type were not observed in the control.

Let us here discuss the information obtained using the Raman confocal microscope to the live tissue analysis. The intensities of Raman bands were measured in the fixed focal volume given by the Raman confocal microscope, and the information obtained there is affected by many factors (superposition of many chemical reactions). Even in the situation it is possible to estimate changes in the concentrations for Raman active chemical species since the intensity of a Raman band is changed in a proportional manner to concentrations. In Figure 4(b), concerning to the Caucasian skin models, the remarkable Raman bands at 1455, 1662, and 1007 cm^{-1} gave intensity changes with synchronicity as function of time, whose bands correspond to the vibrations of the CH_2 , amide, and phenylalanine portions, respectively. The observation of the intensity changes with synchronicity suggests that the concentrations of the Raman active molecules in the fixed focal volume were changed in the same manner. It is probable that the observed dermal cells were changed to different states after the H_2O_2 stimulation to give the synchronous changes in the Raman bands.

In Figure 4(b), the 3D human skin models containing melanocytes exhibited different responses to H_2O_2 . It is of interest that Caucasian keratinocytes commonly used in all the three skin-type models, the majority of the dermal cells, were influenced by the different types of the melanocytes present (the skin models are fabricated by spreading dermal cells on the collagen sheet with the approximate ratio of one melanocyte to twenty keratinocytes; we can model three types of the skin models by choosing Caucasian, Asian, and Black type melanocytes; within melanosomes of a melanocyte, melanin is synthesized and transformed into surrounding keratinocytes through dendrites of the melanocyte; the melanocytes remained in the basal layer

while keratinocytes were proliferated upward in order to form the three dimensionally developed skin models with the basal layer, prickle cell layer, granular cell layer, and keratin layer.) Melanin is a pigment with a complex chemical structure. Two types of melanin coexist in almost all human beings, and their ratio determines an individual's hair and skin color [22, 23]. One is eumelanin, which is brown to black in color, and the other is pheomelanin, yellow to red in color. These are probably the main compounds that cause differences in dermal cells in terms of their sensitivity to H_2O_2 . Melanin is related to the photoprotection of the skin through its ability to absorb and scatter light [22, 23]. Another function of melanin is in the reduction or *generation* of ROS [22, 23]. While ROS reduction is induced by eumelanin, ROS production is done by pheomelanin [22, 23].

4. Conclusion

With H_2O_2 stimulation, the time-dependent Raman spectra of three kinds of skin models exhibited different responses. Changes in the Raman signals were observed at the early stage, compared with the macroscopic pigmentation after stimulation. The properties of the skin models were characterized using the Raman technique. Raman bands for the Caucasian skin model showed large intensity changes within 4–5 days of stimulation, compared to those for the Asian and Black skin model. Eventually, eumelanin and pheomelanin are considered to be the main compounds that differentiate skin properties in terms of their sensitivity to H_2O_2 . The findings are important since the early stage Raman detections of the skin identities may allow us to classify disorders or diseases such as stains, freckles, and cancers of the skin in a newly started manner. Our research group is at present interested in measuring the Raman spectra of single melanocytes or keratinocytes, distinguishing the locations in the target cells to study more specific chemical reactions. Also, we are testing the physical properties of hollow fiber probes to measure Raman spectra of the human skin as it is. These will be discussed elsewhere in the near future.

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