Journal of Dermatological Science 73 (2014) 251–257

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Comprehensive analysis of melanogenesis and proliferation potential of melanocyte lineage in solar lentigines

Takaaki Yamada [a,b,c,*], Seiji Hasegawa [a,b,c], Yu Inoue [a], Yasushi Date [a], Masaru Arima [b], Akiko Yagami [b], Yohei Iwata [b], Masamichi Abe [b], Masayuki Takahashi [b], Naoki Yamamoto [d], Hiroshi Mizutani [a], Satoru Nakata [a], Kayoko Matsunaga [b], Hirohiko Akamatsu [c]

[a] Research Laboratories, Nippon Menard Cosmetic Co., Ltd., 2-7 Torimicho, Nishi-ku, Nagoya, Aichi, Japan
[b] Department of Dermatology, Fujita Health University School of Medicine, 1-98 Kutsukakecho, Toyoake, Aichi, Japan
[c] Department of Applied Cell and Regenerative Medicine, Fujita Health University School of Medicine, 1-98 Kutsukakecho, Toyoake, Aichi Japan
[d] Laboratory of Molecular Biology & Histochemistry, Fujita Health University Joint Research Laboratory, 1-98 Kutsukakecho, Toyoake, Aichi, Japan

ARTICLE INFO

Article history:
Received 26 March 2013
Received in revised form 16 July 2013
Accepted 6 November 2013

Keywords:
Solar lentigines
Melanocyte
Melanoblast
Melanocyte stem cell
Differentiation

ABSTRACT

Background: Solar lentigines (SLs) are characterized by hyperpigmented macules, commonly seen on sun-exposed areas of the skin. Although it has been reported that an increase in the number of melanocytes and epidermal melanin content was observed in the lesions, the following questions remain to be answered: (1) Is acceleration of melanogenesis in the epidermis caused by an increased number of melanocytes or the high melanogenic potential of each melanocyte? (2) Why does the number of melanocytes increase?
Objective: To elucidate the pathogenic mechanism of SLs by investigating the number, melanogenic potential and proliferation status of the melanocyte lineage in healthy skin and SL lesions.
Methods: Immunostaining for melanocyte lineage markers (tyrosinase, MART-1, MITF, and Frizzled-4) and a proliferation marker, Ki67, was performed on skin sections, and the obtained images were analyzed by image analysis software.
Results: The expression level of tyrosinase to MART-1 of each melanocyte was significantly higher in SL lesions than healthy skin. The numbers of melanocytes in the epidermis, melanoblasts in the hair follicular infundibulum and melanocyte stem cells in the bulge region were increased in SL; however, no significant difference was observed in the Ki67-positive rate of these cells.
Conclusion: The melanogenic potential of each melanocyte was elevated in SL lesions. It was suggested that the increased number of melanocytes in the SL epidermis might be attributed to the abnormal increase of melanocyte stem cells in the bulge.

1. Introduction

Solar lentigines (SLs) are characterized by hyperpigmented macules, commonly seen on sun-exposed areas of the skin, such as the face and arms [1,2]. Because of the negative social impact on the quality of life of patients, elucidation of the pathogenic mechanism of SLs is anticipated in order to develop effective treatments. Previously, it has been reported that expressions of some growth factors were up-regulated in the lesion. Major melanogenesis-related factors, endothelin-1 (ET-1) and stem cell factor (SCF), were elevated in the epidermis [3,4], and fibroblasts over-expressed hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) as well as SCF in the dermis [5,6]. Although these growth factors are thought to stimulate melanogenesis in SL lesions, to our knowledge, no studies have analyzed the melanogenic potential of each melanocyte, not the entire epidermis in the lesion [7]. A significant increase in the number of melanocytes in the epidermis was also observed by immunohistological analysis of several melanocytic antigens [1,3,8,9]. The above-mentioned growth factors can also stimulate the proliferation of melanocytes as well as melanogenesis in vitro [10,11]; therefore, these factors are thought to be involved in the increase of melanogenesis and proliferation of melanocytes in SL lesions [12]. However, the proliferation potential of epidermal melanocytes in SLs has hardly been investigated. Although acceleration of melanogenesis of the entire epidermis and an increased number

* Corresponding author at: Research Laboratories, Nippon Menard Cosmetic Co., Ltd., 2-7 Torimicho, Nishi-Ku, Nagoya, Aichi 451-0071, Japan. Tel.: +81 52 531 6263; fax: +81 52 531 6277.
E-mail address: yamada.takaaki@menard.co.jp (T. Yamada).

0923-1811/$36.00
http://dx.doi.org/10.1016/j.jdermsci.2013.11.005

of melanocytes in SL lesions are well-known characteristics of SL pathology, the following questions remain to be answered: (1) Is the acceleration of melanogenesis in the SL epidermis caused by an increased number of melanocytes or the high melanogenic potential of each melanocyte? (2) Why does the number of melanocytes increase?

A previous observation suggested the existence of a melanocyte reservoir in hair follicles [13,14]. Nishimura et al. first demonstrated that melanocyte stem cells (McSCs) existed in the bulge area of hair follicles [15]. When the hair cycle progresses from the resting phase (telogen) to growth phase (anagen), McSCs divide and differentiate into melanoblasts, progenitors of melanocytes, which are immature and unpigmented cells [16,17]. After migrating into hair bulbs, melanocytes further differentiate into mature melanocytes and supply melanins to surrounding keratinocytes. Previous studies on K14-SCF transgenic mice, which produce SCF in epidermal basal keratinocytes, showed that McSCs gave rise to epidermal melanocytes as well as hair bulb melanocytes. We also reported that McSCs expressed Frizzled-4 (FZD4) on their surface and FZD4 was a useful marker to identify and isolate McSCs from murine skin [18]. Therefore, it is important to consider the differentiation process of McSCs into melanoblasts/melanocytes to elucidate the pathogenic mechanism of pigmentary disorders such as SL.

In the present study, we analyzed the tyrosinase expression level of each melanocyte as an indicator of melanogenic potential in healthy skin and SL lesions by image analysis software. Tyrosinase expression levels in each melanocyte were normalized by MART-1. MART-1 is considered to be an appropriate marker to widely analyze melanocyte lineage cells since it is expressed from an earlier stage than tyrosinase [19]. We also analyzed the numbers of melanocyte lineage cells as well as their localization and proliferation potential and further investigated the relationship between SL formation and McSC differentiation.

2. Materials and methods

2.1. Skin biopsies

Samples of SLs and normal skin were collected from 11 subjects for each (SL: 8 women and 3 men, average age 63.6 ± 15.3; normal: 10 women and 1 man, average age 66.7 ± 14.7) at Fujita Health University Hospital and examined by conventional Fontana-Masson staining. After ensuring that the patients fully understood the study objective and other related information, written informed consent was obtained from each subject. This study was approved by the Ethics Committee of Fujita Health University.

2.2. Immunohistochemistry

Formalin-fixed and paraffin-embedded sections prepared from skin biopsies were deparaffinized and boiled in Target Retrieval Solution (Dako, Glostrup, Denmark). After antigen retrieval, the sections were incubated with anti-Ki67 (ThermoScientific, Fremont, CA, USA) and anti-melanoma antigen recognized by T-cells (MART-1; ThermoScientific) antibodies, anti-FZD4 (Millipore, Billerica, MA, USA) and anti-microphthalmia-associated transcription factor (MITF; ThermoScientific) antibodies, or anti-FZD4 and anti-Ki67 (BioLogo, Kronshagen, Germany) antibodies followed by staining with Alexa Fluor 488-labeled anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA) and AlexaFluor 594-labeled anti-mouse IgG. For dual staining for TYR and MART-1, the sections were incubated with anti-TYR antibody (ThermoScientific) followed by staining with Alexa Fluor 488-labeled anti-rabbit IgG, and then incubated with anti-MART antibody labeled with AlexaFluor 594 using a Zenon AlexaFluor 594 mouse IgG labeling kit (Life Technologies). 4′,6-Diamidino-2-phenylindole (DAPI; VECTASHIELD H-1200; Vector Laboratories, Burlingame, CA, USA) was used for nuclear staining.

2.3. Image analysis

Specimens were immunostained and observed by fluorescent microscopy, and microscopic images were collected randomly. Relative fluorescent units (RFU) of TYR and MART-1 in the images were analyzed using NIH Image. The relative TYR expression level of each melanocyte was calculated by dividing the RFU of TYR by the RFU of MART-1 using the following equation. Relative TYR expression level = RFU of TYR/RFU of MART-1. The average value of the relative TYR expression level of each melanocyte was adopted as the relative TYR expression level of a subject. Finally, based on the relative TYR expression level, the average values of the relative TYR expression level of healthy (non-SL) and SL sites were calculated. The number of melanocytes, $Ki67^{+}$ cells and nuclei stained with DAPI in the basal layer were counted, and the percentage of these cells per basal cells was calculated. Fontana-Masson-stained sections were also analyzed using NIH image as previously reported [20], and relative melanin content per unit area was calculated.

2.4. Statistical analysis

Data are presented as the mean ± SD. $P < 0.05$ was considered significant. Statistical analysis was performed using the *t*-test.

3. Results

3.1. Analysis of melanogenesis and proliferation potential of epidermal basal melanocytes

Samples of normal skin and SLs were obtained from groups of similar age subjects (40s–80s). Ten of 11 SL samples were categorized into stage 1 and only one sample was categorized into stage 2 according to the definition by Cario-Andre et al. [1]. Fontana-Masson staining confirmed that the epidermal basal melanin content was significantly higher in SL lesions than in healthy skin (Fig. 1A and B). To analyze the tyrosinase (TYR) expression level of individual melanocytes as an indicator of melanogenesis potential, dual immunohistochemistry was performed for TYR and melanoma antigen recognized by T-cells (MART-1) (Fig. 1C–H). MART-1 was widely expressed by relatively immature melanoblasts and mature melanocytes [21], while TYR, a rate-limiting enzyme of melanogenesis, was rarely expressed by immature melanoblasts. Consistent with previous reports, a significant increase in the number of epidermal melanocytes was observed in SL lesions compared to normal skin (Table 1). In order to quantify the TYR expression level of individual melanocytes in each skin sample, after measuring RFU of MART-1 and TYR by image analysis software, the RFU of TYR was normalized to the RFU of MART-1. MART-1 and TYR are observed in early- and late-stage melanosomes, respectively [19]. This indicates that MART-1 is expressed earlier than TYR, and thus it was considered to be an appropriate marker to widely analyze melanocyte lineage cells, for example, when normalizing the specific protein expression level of each melanocyte. An example of the quantification of the TYR expression level in individual melanocytes is shown in Fig. 1I–K. As a result, TYR expression level of SL melanocytes was significantly higher than normal skin melanocytes (Fig. 1L). These results revealed an increase in the number of melanocytes and acceleration of melanogenesis in SL lesions.