Original Research Article

Pigmentation in Intrinsically Aged Skin of A1 Guinea Pigs

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It is known that skin often shows irregular pigmentation during aging which is frequently associated with hyperpigmentation. Many studies have utilized brownish A1 guinea pigs to investigate the pathogenesis of ultraviolet (UV)-induced skin pigmentation, however, changes associated with intrinsic aging in A1 guinea pig skin have not been documented. To characterize such changes, skin from the dorsal and neck areas of 20-week, 1-, 2-, 3- and 5-yr-old guinea pigs was examined. Skin color was measured using a colorimeter, and biopsy specimens were stained with Masson-Fontana, L-3,4-dihydroxyphenylalanine (DOPA), and antibodies against KIT (ACK-45), gp100 (HMB-45) and S-100 proteins. The $L^*$ value of skin color decreased with aging and melanin deposits increased in the epidermis. Further, DOPA+, gp100+ and S-100+ melanocytes increased, indicating that the number of melanocytes had increased with age, whereas KIT+ melanocytes did not increase in dorsal skin and actually decreased in neck skin with aging. Further, rippled pigmented areas appeared in the neck skin of the 3-yr-old animals, and in the dorsal and neck skin of 5-yr-old guinea pigs in the absence of UV irradiation. Melanocytes were distributed uniformly in younger skin, whereas they were clustered in older skin. UV irradiation caused an increase in the number of melanocytes, although they were not clustered. These results are the first to provide evidence that pigmentation is induced in the skin of intrinsically aged A1 guinea pigs in the absence of UV irradiation, a process that differs from that elicited by UV irradiation.

Key words: Aging, Melanocyte, A1 guinea pig, Pigmentation, Cluster

INTRODUCTION

The total number of melanocytes in human skin decreases with age (1–3), and aged skin often shows irregular pigmentation that is frequently associated with hyperpigmentation (4,5). Solar lentigos are considered hallmarks of older skin and it has been postulated that skin color heterogeneity in ultraviolet (UV)-exposed areas is due to an uneven distribution of pigment cells (5). Microscopic analyses of this type of lesion have shown increases in the number of melanocytes above the dermo-epidermal junction, as well as an increase in melanosomes in surrounding keratinocytes (5,6). However, the mechanism by which pigmentation is induced during aging has not been clarified.

Guinea pigs possess a moderate number of melanocytes and melanosomes in the epidermis, similar to the distribution found in human skin (7), and thus many studies have utilized A1 guinea pigs to investigate the pathogenesis of UV-induced skin pigmentation (8–10). In UVB-irradiated guinea pig skin, the number of melanocytes was previously reported to be increased along with melanin throughout the entire epidermis, while visible pigmentation was also clearly observed (7,10). However, changes associated with aging in the skin of A1 guinea pigs have not been documented.

The average life span of guinea pigs is 4–7 yr (11). In our laboratory, we kept a black guinea pig for approximately 5 yr and incidentally observed irregular pigmentation in its dorsal skin as it aged. We therefore considered that guinea pigs might also be a useful model to understand the irregular pigmentation associated with skin aging.

Abbreviations – $a$-MSH, $a$-melanocyte stimulating hormone; bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; MED, minimum effective dose of radiation; RT, room temperature; SCF, stem cell factor

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In the present study, to investigate such pigmentation, we systematically kept A1 guinea pigs and observed changes in their skin that are associated with aging, using the l-3,4-dihydroxyphenylalanine (DOPA) stain as well as staining with antibodies against gp100 and S-100, specific markers of melanosomes and melanocytes commonly used in melanocyte research (12–15). Further, an anti-KIT antibody was used, because it has been reported that stem cell factor (SCF)/KIT signaling is associated with the development and function of melanocytes (16–19).

**MATERIALS AND METHODS**

**Animals**

Female brownish A1 guinea pigs from 20 weeks to 5 yr old were obtained from Tokyo Jikken Doubutu Inc. (Tokyo, Japan). All guinea pigs had reached sexual maturity, as the age of sexual maturity is 2 months, while the estrous cycle is 16 d. Guinea pigs were divided by age into groups of 20-week-old (n = 6), 1-yr-old (n = 7), 2-yr-old (n = 6), 3-yr-old (n = 5) and 5-yr-old (n = 5) animals, and were housed in individual cages with free access to food and water, with the environmental temperature maintained at 22 ± 1°C and a relative humidity of 55 ± 5%. Animal husbandry procedures were in accordance with the Guidelines for Animal Experiments (Japan Association for Laboratory Animal Science, 1987).

**UV Irradiation**

UV irradiation was administered using an FL32S-E-30DMR lamp (Toshiba, Tokyo, Japan). The energy emitted from the lamps was within the UVB range (280–320 nm). Twenty-week old guinea pigs (n = 6) were shaved with electric clippers to remove long hair and the remaining stubble was removed with a shaver. They were irradiated once and received a total of 500 mJ/cm2. The minimum effective dose of radiation (MED) for guinea pigs is 200–300 mJ/cm2; thus the 500 mJ/cm2 is considered to be approximately equivalent to 2 MED. On the 14th day after UV irradiation, skin samples were taken.

**Measurement of Skin Color**

Prior to the measurement of skin color, skin at the dorsal and neck areas were shaved with electric clippers to remove long hair and the remaining stubble was removed with a shaver. Skin color intensity was measured at 10 sites using a Color Reader CR-11 MUNSELL (Minolta, Osaka, Japan) and is expressed as L*, a* and b* values, with the C* value calculated from those. The L* value (luminance) represents brightness ranging from total black (lowest value) to total white (highest value), while the a* value represents the color balance from green (lowest value) to red (highest value), and the b* value is the color balance from blue (lowest value) to yellow (highest value). The C* value (luminance) represents color from gray (lowest value) to vivid (highest value), and was calculated as $C^* = [(a^*)^2 + (b^*)^2]^{1/2}$.

**Masson–Fontana Staining**

Specimens from dorsal and neck skin were frozen in embedding medium (Tissu Mount; ChibaMedical Inc., Saitama, Japan), cut into 5 μm-thick sections, placed on glass slides (Matsunami, Tokyo, Japan), and stained with Masson–Fontana stain.

**Preparation of Epidermal Sheets**

Skin samples (0.5 × 0.5 mm) were taken from the dorsal and neck areas of normal guinea pigs, and from the backs of guinea pigs 14 d after UV exposure. The samples were incubated in 2 N NaBr for 2 h at 37°C, which attached epidermal side down to Double-Sticky Discs (3M, Borken, Nordrhen-Westfalen, Germany) affixed to glass slide surfaces and the dermis was manually separated, exposing the basal epithelial surface.

**DOPA Stain**

After washing the epidermal sheets with phosphate-buffered saline (PBS), they were incubated in 0.1% L-3, 4-DOPA (Sigma, St Louis, MO, USA) in PBS for 6 h at 37°C, with the solution changed every 1.5 h. The epidermal sheets were washed in distilled water, then dehydrated with graded alcohol and cleared in xylene. Hairs were removed from the sheets and they were mounted with Malinol (Muto Purd Chemical Ltd, Tokyo, Japan).

**Immunohistology**

To investigate the number and distribution of melanocytes in each different developmental stage, we used the following antibodies: KIT as a marker of early or differentiated melanocytes (20), HMB-45 recognizes early melanosomes (21) and S-100 protein reacts with neural or neural crest tissues, including melanocytes (22).

**KIT Immunostaining**

Epidermal sheets were washed with PBS and fixed in acetone for 15 min at 4°C, then, after washing again in PBS, stored at −80°C. The sheets were defrosted in PBS and incubated in normal goat serum for 20 min to block non-specific staining, after which they were incubated with a primary antibody, rat IgG-anti-mouse ACK45 monoclonal antibody (1:100, BD Bioscience, Franklin Lake, NJ, USA), for 1 h at room temperature (RT), followed by incubation with alkaline phosphate conjugated with anti-rat-IgG + IgM (1:100, Southern Biotechnology Associates, Inc., Birmingham, AL, USA). Bound antibodies were detected with the New Fuchsin Substrate System (DAKO, Carpinteria, CA, USA) and a levamisole solution (Vector, Burlingame, CA, USA) as an inhibitor of internal alkaline phosphatase. Sheets were mounted with Glycergel (DAKO).

**gp100 Immunostaining**

Epidermal sheets were washed with PBS and fixed in acetone for 15 min at 4°C, then, after washing again in PBS, stored at
The sheets were defrosted in PBS and incubated in 3% H₂O₂ for 10 min to block endogenous peroxidases. After incubation with the primary antibody, mouse–anti-human HMB-45 monoclonal antibody (1:100, DAKO), for 24 hr at 4°C, the sheets were washed with PBS. Bound antibodies were detected using the avidin-biotin peroxidase method (LSAB2 Kit; DAKO). The sheets were then reacted with biotinylated anti-mouse immunoglobulins for 10 min at RT and the color was developed using 3-amino-9-ethylcarbazole (AEC; DAKO). The sheets were mounted with Glycergel (DAKO).

S-100 Immunostaining

Epidermal sheets were fixed in 4% paraformaldehyde for 20 min at RT, then, after washing with PBS, stored at −80°C. The sheets were defrosted in PBS and incubated in 3% H₂O₂ for 10 min to block endogenous peroxidase. After incubation with the primary antibody, anti-S-100 polyclonal antibody (1:1000, Affiniti Research Products Ltd, Exeter, Devon, UK), for 24 h at 4°C, the sheets were washed with PBS. Bound antibodies were detected using the avidin-biotin peroxidase method (LSAB2 Kit; DAKO). The sheets were then reacted with biotinylated anti-rabbit immunoglobulins.

Fig. 1. Pigmentation with intrinsic aging. Representative photographs of dorsal skin (A–C) and neck skin (D–F) from 20-week old (A, D), 3-yr old (B, E) and 5-yr old (C, F) A1 guinea pigs.

Fig. 2. Changes of skin color with intrinsic aging. Shown are changes of L* value in dorsal (A) and neck (B) skin. The L* value (luminance) represents brightness ranging from total black (lowest value) to total white (highest value). Also shown are changes of a*, b* and C* values in dorsal (C) and neck (D) skin. The a* value (luminance) expresses the color balance from green (lowest value) to red (highest value), the b* value (luminance) expresses color balance from blue (lowest value) to yellow (highest value), and the C* value (luminance) expresses color from gray (lowest value) to vivid (highest value). The C* value was calculated as $C^*=[(a^*)^2+(b^*)^2]^{1/2}$. Values are shown as the mean ± SE (20-week and 2-yr-old, n = 6; 1-yr-old, n = 7; 3- and 5-yr-old, n = 5). *P < 0.05; **P < 0.01; ***P < 0.001 Dunnett multiple test (vs. 20-week-old).
Fig. 3. Melanin distribution with aging. Masson–Fontana staining was performed using skin samples from 20-week old (A, D), 3-yr old (B, E) and 5-yr old (C, F) A1 guinea pigs. Both dorsal (A–C) and neck skin (D–F) areas were tested. In young epidermis, melanin was rarely observed, however, with aging, it was seen throughout the entire thickness of the epidermis, including the stratum corneum. Scale bar 100 μm.

Fig. 4. Melanocyte clustered with intrinsic aging. Epidermal sheets were prepared from dorsal (A–E) and neck skin (F–J) specimens, and stained with anti-gp100 antibodies. Shown are representative photographs of melanocytes from 20-week old (A, F), 1-yr old (B, G), 2-yr old (C, H), 3-yr old (D, I) and 5-yr old (E, J) A1 guinea pigs. Melanocytes became more prominently clustered in the pigmented areas with age. Scale bar 100 μm.
for 10 min at RT and the color was developed using 3-amino-9-ethylcarbazole (AEC; DAKO). The sheets were mounted with Glycergel (DAKO).

Counting of Melanocytes

The numbers of DOPA+, KIT+, gp100+ and S-100+ melanocytes per mm² were counted using a light microscope (Nikon, Tokyo, Japan) at a magnification of 200 ×.

Statistical Analysis

Statistical analysis was performed using the Kruskal–Wallis test or the Dunnett multiple comparison test.

RESULTS

Changes in Pigmentation of A1 Guinea Pig Skin with Aging

Using all guinea pigs from each age group, skin in the dorsal and neck areas were shaved in order to observe changes in pigmentation by the naked eye. In young guinea pig skin, no pigmentation was seen in the dorsal and neck areas (Fig. 1A, D), whereas rippled pigmentation appeared in the dorsal areas of 5-yr-old guinea pigs (Fig. 1C). Further, rippled pigmentation appeared in the neck areas of 3-yr old guinea pigs, which became more prominent in 5-yr-old animals (Fig. 1E, F).

The skin color intensity of those areas was measured using a color reader. Luminance ($L^*$) was significantly decreased with aging. In the dorsal skin, $L^*$ was significantly decreased...
in 3-yr-old animals (Fig. 2A), and, in the neck skin, it was significantly decreased in 2-yr-old animals (Fig. 2B). In all animals, the overall color of the neck skin was darker than the dorsal skin. Further, luminance \( (a^*) \), \( (b^*) \), and \( (C^*) \) did not significantly decrease with aging (Fig. 2C, D).

Frozen sections from biopsies of those guinea pigs were stained for melanin using Masson–Fontana. Melanin deposits were rarely observed in the epidermis of 20-week-old guinea pigs (Fig. 3A, D), however, with aging, melanin appeared in the basal epidermal layer and was seen throughout the epidermis, including the stratum corneum. Notably, melanin was abundant in epidermal specimens from 3- and 5-yr-old guinea pigs (Fig. 3B, C, E, F). The epidermis tends to become thinner with age, though not significantly, thus it is possible that melanin accumulation in the epidermis with aging may result from the delay of epidermal turnover known to occur with aging.

**Melanocytes Concentrate and Increase with Aging**

DOPA+, gp100+, S-100+ and KIT+ melanocytes were observed in the epidermal sheets obtained from all guinea pigs, and the distribution was correlated with pigmentation. Melanocytes seemed to line up in the same direction and run parallel to the hair follicles. In the dorsal skin, melanocytes were distributed uniformly between the lines of hair follicles in the 20-week-old guinea pigs (Fig. 4A, Fig. 5A–D), while they were arranged in a striped pattern in the 2-yr-old animals (Fig. 4C) and were thicker in the 3-yr-old animals (Fig. 4D). In 5-yr-old guinea pigs, melanocytes were gathered and clustered slightly (Figs 4D, E and 5E–H). In the neck skin, the clustering of melanocytes occurred more rapidly than in the dorsal skin (Fig. 4F–J).

Further, melanocytes were also increased in UV-irradiated guinea pig skin, however, they were uniformly distributed and were not clustered (Fig. 6).

The numbers of melanocytes in these epidermal sheets were counted (Fig. 7). In the dorsal skin, the numbers of DOPA+, gp100+ and S-100+ melanocytes increased with aging, whereas the number of KIT+ melanocytes did not change. In the neck skin, the numbers of DOPA+, gp100+ and S-100+ melanocytes increased with aging, whereas the number of KIT+ melanocytes decreased, a decrease that was statistically significant in skin from the 5-yr-old guinea pigs.

**DISCUSSION**

A variety of hyperpigmented conditions are seen in human skin (23, 24), although the mechanism(s) by which pigmentation is induced during aging has not been clarified. In the present study, rippled pigmentation appeared on the dorsal and neck areas of intrinsically aged A1 guinea pigs, and melanocytes were clustered and increased in number. Our findings provide the first evidence that increased numbers of clustered melanocytes cause irregular pigmentation in the skin of aged A1 guinea pigs.

To investigate the number and distribution of melanocytes, we used antibodies against KIT and other melanocyte markers (S-100 and HMB-45), in addition to the DOPA reaction. KIT is a marker of early or differentiated melanocytes (20), while DOPA and HMB-45 are related to tyrosinase activity and melanosome stage, respectively (21, 25). Further, tissues containing S-100 protein are either neural or
neural crest derivatives, including melanocytes (22). The present results show that the total numbers of S-100+, DOPA+ and gp100+ melanocytes increase in the skin of aged A1 guinea pigs. We speculate that the same growth factors released in aged guinea pigs may also be found in humans. In fact, human melanocytes require synergistic mitogens in addition to common growth factors present in the serum in order to proliferate in vitro (26). Peptide growth factors known to stimulate melanocyte growth include fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), SCF, endothelins and α-melanocyte stimulating hormone (α-MSH), and their cognate receptors are likely to be important in vivo as well (26). We speculate that such growth factors might also be released in the skin of aged guinea pigs. In contrast, the number of KIT+ melanocytes did not increase in dorsal or neck skin and actually decreased in the neck skin of aged guinea pigs. Several reports have demonstrated that UVB irradiation increases the expression of SCF in human epidermis (18), that KIT+ melanocytes in mouse epidermis decrease following UV exposure (19), and that KIT molecules are internalized and degraded after being activated by SCF in vitro (27). In these guinea pigs, we found that the number of KIT+ melanocytes decreased 1 d after UV irradiation, and then recovered by day 3 and subsequently increased (data not shown). It was also reported that SCF injections induce an increase in the number of melanocytes and hyperpigmentation in human subjects (16). Therefore, the KIT/SCF pathway may have an influence on the relationship of melanocytes with pigmentation in the skin as a consequence of aging, in addition to UV irradiation. The process involved with the increase of melanocytes with aging might be similar to that caused by UV irradiation.

We also found that melanocytes are clustered in aged guinea pig skin, while no clustering was observed in the UV-irradiated specimens, indicating that melanocyte clustering is specific to intrinsically aged A1 guinea pigs. Guinea pigs (which belong to the rodent family) have parallel rows of hair follicles, as occurs in mice and rats which also belong to that family (28). These clustered melanocytes seen our observations seemed to stand in the same direction, running parallel to the lines of hair follicles. It has been reported that immature melanocytes are located along the hair in the outer root sheath and in the hair follicle bulge (29, 30), and that their migration from the hair-follicle outer root sheath into clinically depigmented epidermis is crucial for the repigmentation of vitiliginous skin (31, 32). Thus, it is possible that immature melanocytes in the outer root sheath of the hair follicle migrate into the skin and differentiate as they mature, resulting in their clustering.

As for the increase of melanocytes, the stimulation processes of aging and UV irradiation were similar, because an increase in the number of melanocytes was observed in aged guinea pigs and in those that received UV irradiation. However, in aged guinea pigs, we also observed clustering of melanocytes. Thus, in the process of aging, it is possible that another factor is involved with the accumulation of melanocytes. Further investigation of the effects of aging is required to resolve this issue.

In humans, older skin and solar lentigos frequently show irregular pigmentation associated with hyperpigmentation (5, 6). Melanocytes are increased in number in lentiginous epidermis (33, 34), and we observed that the number of melanocytes was increased in the pigmented skin of these aged guinea pigs. However, we also observed that the clustered melanocytes stand in the same direction and run parallel to the lines of hair follicles, which has not been reported in lentigo. Further, previous studies have found a downward budding of pigmented basal cells in lentiginous epidermis (35, 36), whereas such downward budding was not observed in the pigmented skin of the intrinsically aged A1 guinea pigs in this study. In humans, pigmentation mostly occurs in exposed skin, while it is also observed in non-exposed aged guinea pig skin. Therefore, we speculate that the downward budding seen in solar lentigos may be due to effects of UV irradiation, for example, the destruction of the basement membrane structure (36). Our results indicate that pigmentation in these aged A1 guinea pigs is not consistent
with the pigmentation in lentigens of humans. Further investigation of the irregular pigmentation that occurs in aged guinea pig skin may provide clues to the mechanisms of pigmentation. Investigation of the effects of UV irradiation on guinea pig skin during aging is required.

In conclusion, our findings demonstrate that an increase in number and in the clustering of melanocytes occurs in the hyperpigmentation induced in the skin of intrinsically aged A1 guinea pigs, and that this process differs from the UV-induced pigmentation.

REFERENCES
