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Original article

Melanin synthesis induction by advanced glycation end-products (AGEs) without α-melanocyte stimulating hormone (α-MSH) or UV exposure

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Abstract

Ultraviolet (UV) radiation causes irregular skin pigmentation known as freckles. The mechanism behind UV-induced melanin production has been studied extensively, with the resulting knowledge being used to develop commercial skincare products. Some freckles, such as senile lentigo (age spots), may appear independently of UV exposure. The role of aging in the development of these freckles is not well-studied. We hypothesized that advanced glycation end-products (AGEs) induce age spots and, therefore, examined their effects on melanogenesis in B16 murine melanoma cells (B16F10). Significant melanin production occurs in melanoma cells, after incubation with AGE-collagen (collagen-glucose or collagen-fructose). We further investigated the active compounds in AGE-collagen and identified methylglyoxal (MGO) as having the strongest activity. But melanin production by collagen-fructose is less active than collagen-glucose, even though it contains more MGO. This discrepancy suggests that AGEs-induced melanogenesis is caused by multiple factors. This is the first report of melanin synthesis being stimulated by AGEs without exposure of UV orα-melanocyte stimulating hormone (α-MSH). As AGEs-induced melanogenesis can result in brown spots or freckles on the skin (AGE pigment freckle), managing AGEs may be as important as UV care for the maintenance of healthy, bright skin.

KEY WORDS: Advanced glycation end-products (AGEs), AGE pigment freckle, collagen, glucose, fructose, B16 melanoma cell.

Introduction

Aging deteriorates cellular function and structure and frequently leads to organ dysfunction. Morphological changes in skin are obvious. As such, skin aging receives a lot of medical and commercial attention. With increasing age, dermal and epidermal layers become thin, less resilient, and prone to damage. While such changes seem inevitable, the skin is often more affected by environmental factors than genetics. Among these factors, ultraviolet (UV) irradiation is the most deleterious and is responsible for photoaging [1, 2]. Photoaging is defined as a "characteristic morphological change such as wrinkles and freckles induced by chronic UV exposure" [2]. However, senile lentigo may also develop independently of UV exposure. The cause is still unknown, but it is likely due to age-related decline in, and/or abnormal cellular function.

Glycation stress has been reported as a skin aging factor [3]. It results from the accumulation of advanced glycation end-products (AGEs) that are formed by non-enzymatic reactions between reducing sugars and proteins. The accumulation of AGEs in tissues induces a number of structural and functional changes, including skin aging. Glycated collagens lose their resilience, leading to wrinkles and inflexible skin [4]. Ogura *et al.* reported that lipid peroxidation-induced protein carbonylation was responsible for dull and yellowish skin tones [5]. However, it is not known if AGEs or glycation stress contributes to freckling. Therefore, we investigated the role of AGE accumulation on melanogensis in B16 murine melanoma cells.

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Materials and methods

Reagents

Synthetic melanin, [Nle^4, D-Phe^7]-α-melanocyte stimulating hormone trifluoroacetate salt (α-MSH), 3, 4-Dihydroxy-L-phenylalanine (L-DOPA) and methylglyoxal (MGO) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Glyoxal (GO) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-Deoxyglucose (3DG) was purchased from Dojindo Laboratories (Kumamoto, Japan). N^{ε}-(carboxymethyl) lysine (CML), N^{ε}-(carboxyethyl) lysine (CEL) and pentosidine were purchased from Kurabo Industries Ltd. (Osaka, Japan). Collagen type I (bovine skin, pepsin-solubilized) was purchased from Nippi, Incorporated (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich (Tokyo, Japan).

Preparation of AGE-modified collagen

AGE-modified collagen (AGE-collagen) was prepared according to the method of Hori *et al.*[6], with minor modifications. Briefly, the reaction solution contained 250 μL of 200 mM phosphate-buffer (PB; pH 7.4), 200 μL of 2.0 M sugar (glucose; Glu or fructose; Fru) solution, 200 μL of 3.0 mg/mL collagen and 350 μL water. Control solutions were prepared without collagen and/or without sugar. Then the reactions were incubated at 60 °C for 0, 1, 10, 24, 72 and 168 h. When conducting a test, reaction samples were diluted to 20 times in DMEM.

Fluorescence AGEs measurement

The fluorescence intensity of AGEs was measured with a microplate reader (Thermo scientific Varioskan™ Flash Multimode Reader, Thermo Fisher Scientific K.K., Kanagawa, Japan) at an excitation of 370 nm and emission at 440 nm. Each fluorescence value was calculated as a ratio between the fluorescence intensity of the sample and 5 μg/mL quinine sulfate in 0.1 N sulfuric acid, multiplied by 1,000.

Quantification of 3-deoxyglucose (3DG), methylglyoxal (MGO) and glyoxal (GO)

3DG, MGO and GO were measured by HPLC according to Hori *et al.*[6], with slight modification. Briefly, 200 μL of reaction solution was combined with 130 μL water and 170 μL 6% perchloric acid, stirred, then centrifuged at 13,800 × g (12,000 rpm) for 10 min. Next, 400 μL of the supernatant was combined with 350 μL saturated sodium bicarbonate solution and 50 μL 2, 3-diaminonaphthalen (DAN) labeling reagent (1mg/mL), stirred, then incubated for 24 h. Afterwards, chromatographic separation involved a Unison UK-Phenyl, 75 × 3 mm I.D., column (Imtakt, Kyoto, Japan). The mobile phase was 50 mM phosphoric acid: acetonitrile (89:11) and was eluted at 1.0 mL/min. Detection was at excitation wavelength 271 nm and detection wavelength 503 nm. Concentrations of 3DG, MGO and GO in samples were determined with standard curves, based on peak areas from previously analyses of chemical reference standards.

Cell culture

B16 murine melanoma cells (B16F10) were purchased from Riken BioResource Center (Ibaragi, Japan) in February 2015. The B16 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS, Nichirei Biosciences Inc., Tokyo, Japan) and 1% Pen Strep (a mixture of 10,000 U/mL penicillin and 10,000 μg/mL streptomycin, Life Technologies Japan Ltd., Tokyo, Japan) at 37°C in a humidified, CO_2-controlled (5%) incubator.

Melanin content assay

Melanin synthesis in B16 cells was evaluated according to methods described by Masuda *et al.*[7] and Lee *et al.*[8], with slight modification. Briefly, 900 μL cell culture in phenol red free DMEM were seeded on 24 well-plates (2×10^4 cells/well), then treated with 100 μL sample 24 h after seeding.

The samples evaluated were AGE-collagen and its chemical components, including MGO, GO, 3DG, CML, CEL and pentosidine. α-MSH was used as the positive control. α-MSH was dissolved in aqueous acetic solution (5%, v/v), then diluted with DMEM to 0.1 μM.

After treatment for 72 h, the cell cultures were centrifuged. The supernatants were collected and transferred to a 96 well-plate for measurement of secreted melanin. Cell pellets were washed with phosphate-buffered saline (PBS, pH 7.4) and lysed with 1N NaOH for 1 h at 60 °C. After centrifugation, the supernatants of lysed cell pellets were transferred to a 96 well-plate for measurement of intracellular melanin. Absorbance was measured at 405 nm with using a microplate reader (Thermo scientific Varioskan™ Flash Multimode Reader, Thermo Fisher Scientific K.K., Kanagawa, Japan), and melanin concentration was determined using a standard curve prepared with synthetic melanin.

Tyrosinase activity assay

Intracellular tyrosinase activity in B16 cells was evaluated according to the slightly modified methods of Masuda *et al.*[7] and Li *et al.*[9]. Cells were seeded and α-MSH used as a positive control, as described above. After treatment for 72 h, the cells were washed with ice-cold phosphate-buffered saline (PBS, pH 6.8) and then lysed with 0.1% triton in PBS (pH 6.8). The lysates were centrifuged at 10,000 rpm and 4 °C for 10 min to obtain a supernatant that contained tyrosinase. Protein concentration of the supernatant was quantified by DC protein assay (Bio-Rad Laboratories, Tokyo, Japan) and adjusted with lysis buffer. The reaction mixture, containing the supernatant (tyrosinase) and 0.1% L-DOPA solution in PBS, was incubated at 37 °C for 20 min. After incubation, dopachrome formation was assayed by measuring absorbance at 475 nm with a microplate reader. Tyrosinase activity in treated cells was calculated as a percentage with respect to activity in pretreated cells.

Cell viability assay

Cell viability was determined by the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), which measures dehydrogenase activity using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfpphenyl)-2*H*-tetrazolium (WST-8) as a substrate. Test samples were dissolved DMEM to an appropriate concentration. In the control group, DMEM solution was used instead of the sample solution. Briefly, 2.8×10^3 cells in the DMEM (90 μL) were placed on 96-well plate. After 24 h of incubation at 37°C, 5% CO_2, AGEs or α-MSH in DMEM (10 μL) were added. After incubation

for 72 h more, DMEM was replaced with a WST solution (WST: culture medium = 1:10, 100 μL). After incubation for 1 h, the resulting formazan was determined by measuring absorbance at 450 nm with a microplate reader. Cell viability was expressed as a percentage of the control group value.

Statistical analysis

The mean ± standard deviation (SD) was calculated for the data obtained from experiments. Intergroup differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using Statcel Ver. 3 (OMS Publishing Inc., Saitama, Japan, 2011).

Results

Effect of AGE-collagen (collagen-glucose or -fructose) on melanogenesis in B16 murine melanoma cells

We investigated the conditions of AGE-induced melanogenesis in B16 murine melanoma cells. Both collagen-Glu and collagen-Fru induced melanin formation after 72 h incubation without affecting cell proliferation (***Fig. 1***). The photoemission from fluorescent AGEs may confound melanin absorption. However, the absorbance of AGE-collagen after 72 h treatment at 37 ˚C was less than 0.02 at 405 nm (data not shown). Therefore, AGEs did not influence the quantification of melanin. Compared to collagen-Fru, collagen-Glu promoted

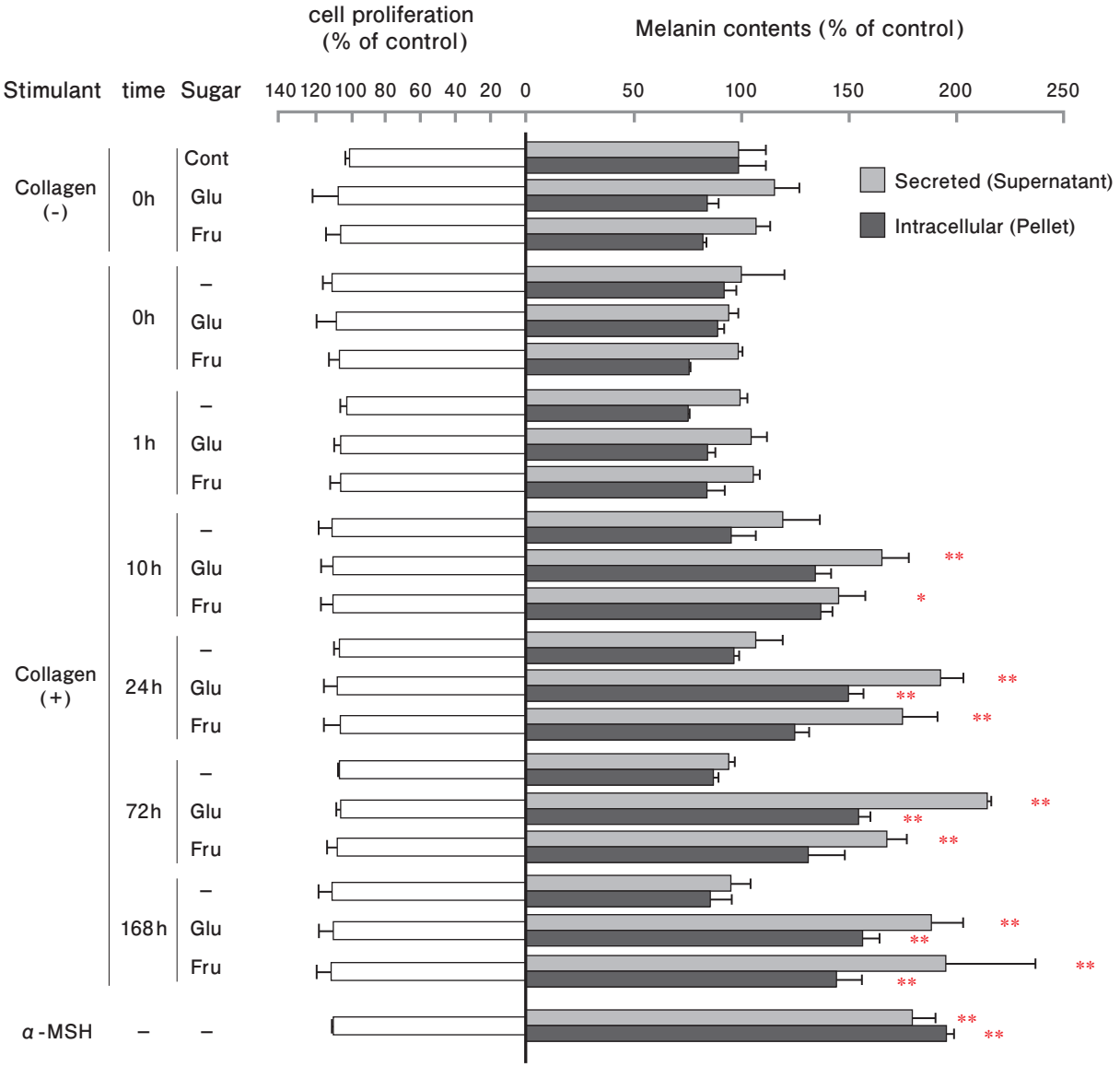

Fig. 1. **Effect of AGE-collagen on melanin synthesis in B16 cells**

Melanin content is provided as the mean ± SD of 3 experiments. Significantly different from the control group: *; $p<0.05$, **; $p<0.01$. Cell proliferation values are mean ± SD of 3 experiments. AGE, advanced glycation end-product; Cont, control; Glu, glucose; Fru, fructose; α-MSH, α-melanocyte stimulating hormone; SD, standard deviation.

melanin formation in a shorter amount of time. Collagen-Glu, incubated for over 24 hours, was able to stimulate melanin production as much as α-MSH. When incubated for over 7 days, collagen-Fru also stimulated melanocytes as much as α-MSH did.

Effect of AGE-collagen (collagen-glucose or-fructose) on tyrosinase activity of B16 murine melanoma cells

Since AGE-collagen stimulated melanin production, we measured tyrosinase activity in B16 murine melanoma cells. While α-MSH significantly enhanced tyrosinase activity, AGE-collagen only had a minor effect (***Table 1***).

Effect of AGE-collagen (collagen-glucose or-fructose) on melanogenesis of B16 murine melanoma cells

We further investigated the active principle responsible for AGE-collagen-induced melanogenesis. Melanogenesis capacity was evaluated for three AGEs (CML, CEL, and pentosidine) and three AGE intermediates (3DG, GO, MGO). CML, CEL, and pentosidine had no activity, while 3DG and GO displayed weak activities. However, MGO exhibited strong melanogenic activity (***Fig. 2***).

Table 1. Effect of AGE-collagen on tyrosinase activity in B16 cells

Stimulant		Tyrosinase activity (%) ($n=3$)	Cell proliferation (%) ($n=3$)
Glu or Fru	Time (h)		
Incubation without collagen			
Control	0	100.0 ± 29.4	100.0 ± 2.8
Glu	0	121.2 ± 7.2	106.8 ± 14.6
Fru	0	120.7 ± 6.2	105.7 ± 8.0
Incubation with collagen			
–	0	101.1 ± 0.7	110.2 ± 5.3
Glu	0	116.4 ± 12.9	107.7 ± 11.8
Fru	0	117.1 ± 14.9	106.3 ± 6.0
–	1	108.7 ± 0.7	102.3 ± 3.5
Glu	1	126.7 ± 4.2	105.7 ± 3.8
Fru	1	118.0 ± 5.6	105.7 ± 5.9
–	10	109.1 ± 1.6	110.1 ± 7.8
Glu	10	126.7 ± 20.9	109.6 ± 7.2
Fru	10	128.9 ± 14.1	109.5 ± 7.2
–	24	114.4 ± 4.8	106.0 ± 3.4
Glu	24	131.4 ± 9.8	107.4 ± 7.8
Fru	24	130.8 ± 14.0	105.7 ± 9.4
–	72	117.5 ± 4.2	105.9 ± 1.2
Glu	72	131.7 ± 8.5	105.4 ± 2.7
Fru	72	122.3 ± 13.7	107.4 ± 6.2
–	168	113.2 ± 1.0	110.3 ± 7.7
Glu	168	122.6 ± 13.4	109.6 ± 8.1
Fru	168	120.3 ± 5.2	110.6 ± 8.7
α-MSH			
–	–	220.7 ± 12.7**	110.0 ± 0.6

Significantly different from the control group, **; $p<0.01$. Results are expressed as mean ± standard deviation. AGEs, advanced glycation endo-products; Glu, glucose; Fru, fructose; α-MSH, α-melanocyte stimulating hormone.

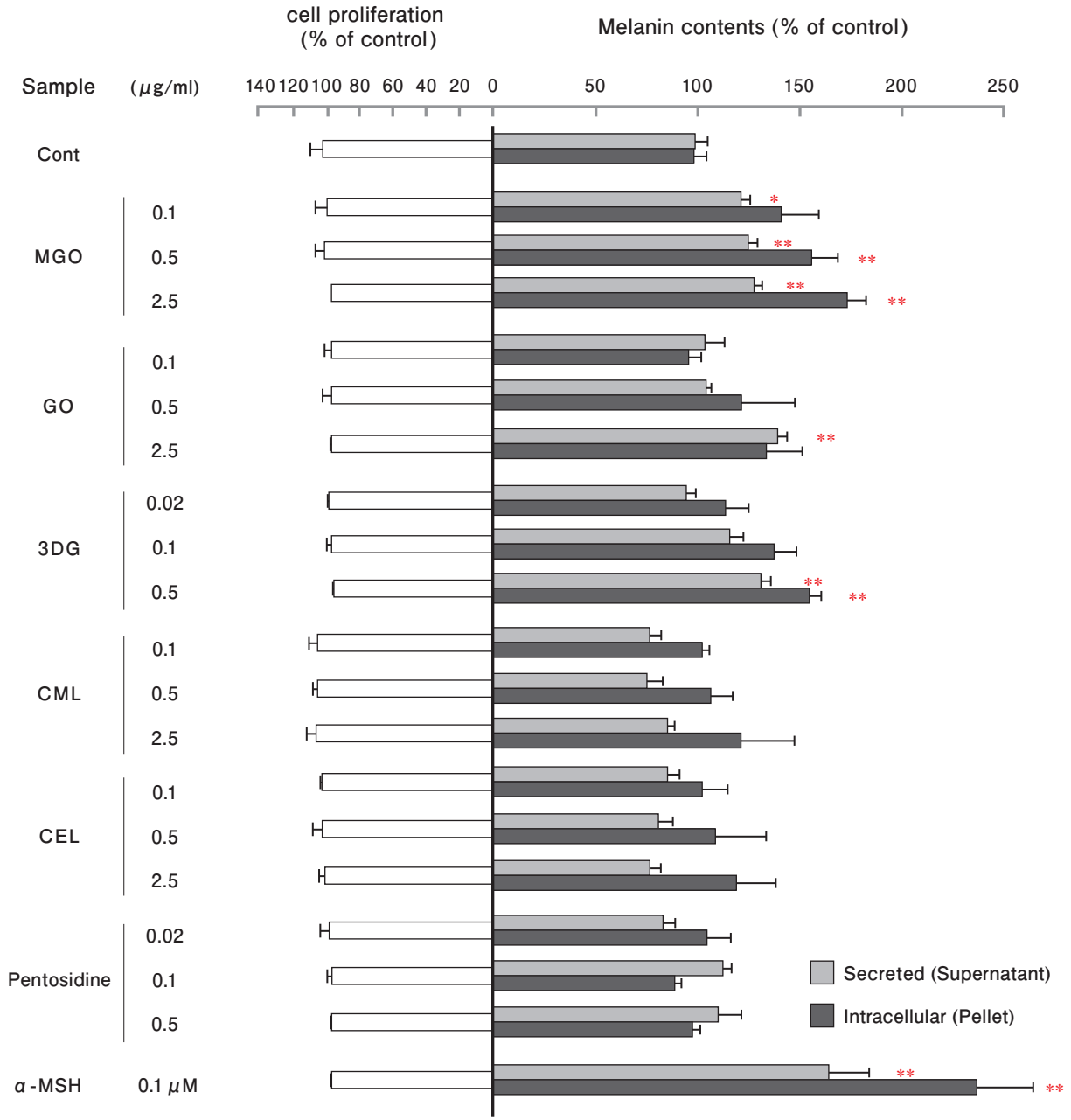

Fig. 2. **Effect of AGEs (CML, CEL and pentosidine) and reaction intermediate (MGO, GO and 3DG) on melanin synthesis in B16 cells**

Melanin content is provided as the mean ± SD of 3 experiments. Significantly different from the control group: *; $p<0.05$, **; $p<0.01$. Each value in cell proliferation represents the mean ± SD of 3 experiments. AGEs, advanced glycation end-products; CML, N^{ε}-(carboxymethyl) lysine; CEL, N^{ε}-(carboxyethyl)lysine; MGO, methylglyoxal; GO, glyoxal; 3DG, 3-deoxyglucosone; Cont, control; α-MSH, α-melanocyte stimulating hormone; SD, standard deviation.

Measurement of fluorescent AGEs, 3DG, MGO and GO in the AGE-modified collagen

We then quantified the formation of fluorescent AGEs and AGE intermediates in AGE-collagen. Crossline, pyrrolopyridine, and pentosidine are examples of fluorescent AGEs. AGE intermediates and fluorescent AGEs occurred less and were more slowly formed in collagen-Glu than in collagen-Fru (***Fig. 3***).

Discussion

Melanogenesis causes irregular pigmentation, such as skin blotches and freckles. Stressors, such as UV light and inflammation, stimulate the production of α-MSH, endothelin-1 (ET-1), stem cell factor (SCF), and secretion of other cytokines in epidermal keratinocytes. As melanocytes react with these activating factors, cellular signals initiate melanin production[10]. UV induced mutation of these genes in keratinocytes may

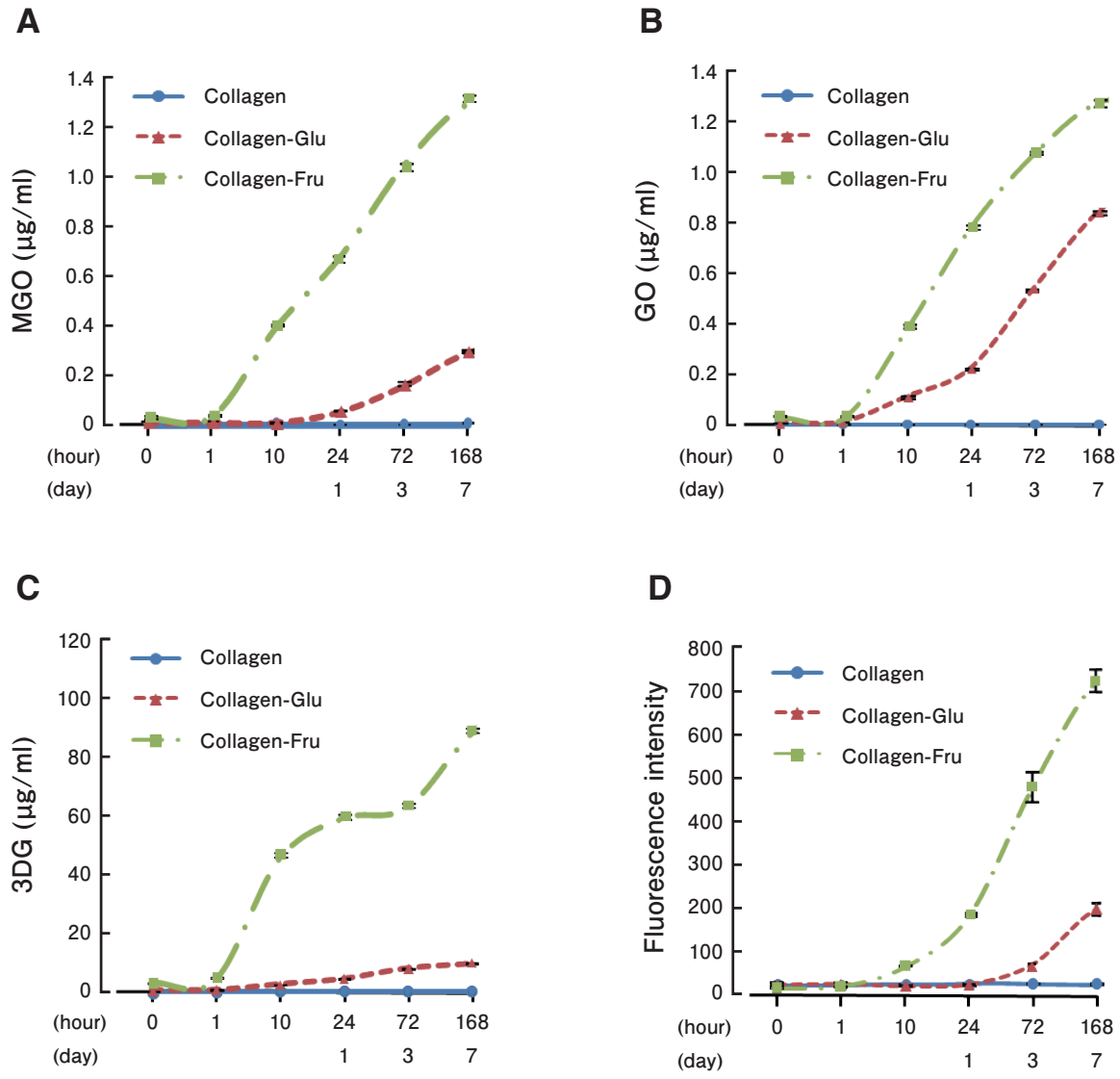

Fig. 3. **MGO, GO, 3DG and fluorescent AGEs in AGE-modified collagen**

A: MGO, **B**: GO, **C**: 3DG, **D**: fluorescent AGEs. Each value represents the mean ± SD of 3 experiments. MGO, methylglyoxal; GO, glyoxal; 3DG, 3-deoxyglucosone; AGEs, advanced glycation end-products; Glu, glucose; Fru, fructose; SD, standard deviation.

cause freckles on the skin. Tyrosinase generates dopaquinone by oxidizing tyrosine. Dopaquinone then undergoes non-enzymatic reactions which lead to precursors of melanin formation. Tyrosinase inhibition has been a main target of the cosmetic industry for the prevention of dark spots or freckles [11]. Many types of skincare products have been developed through the in vitro evaluation of ingredients for inhibitory activity against tyrosinase, melanin production, and α-MSH stimulated gene expression.

In our study, we discovered that AGE-collagen is capable of stimulating melanogensis in B16 murine melanoma cells, with equal or greater activity than α-MSH. This suggests that AGE accumulation may stimulate melanin synthesis and possibly contribute to freckle formation on the skin. However, AGE accumulation only slightly increased tyrosinase activity, an effect which was not statistically significant.

Since over expression of the receptor for advanced glycation end products (RAGE) is reported in various cancer cells [12], B16 murine melanoma cells (B16F10) may also over-express RAGE. AGEs/RAGE interaction reportedly elevates ROS levels through RAGE-NADPH oxidase activation [13, 14]. UV-induced free radical/reactive oxygen species (ROS) production is strongly correlated with melanin content the skin [15], ROS, such as secreted hydrogen peroxide, cause AGE formation [1], including CML [16]. As such, it is possible that melanin production was enhanced by ROS that were generated by RAGE-AGEs interaction. But further study is necessary to confirm this hypothesis.

Wondrak *et al*. reported that UV exposure generates hydrogen peroxide and hampers cellular proliferation of

keratinocytes and fibroblasts. A combination of UV exposure and AGE accumulation synergistically accelerates hydrogen peroxide generation and inhibits cellular growth [1]. The accumulation of AGEs may not be the sole cause of skin damage. But it may be a synergistic enhancer. Epidermal keratinocytes are differentiated regularly and are replaced by new cells. This epidermal turnover causes the excretion of melanin from the epidermis. But AGEs increase integrin expression which slows turnover [17]. AGEs deteriorate healthy skin via three potential mechanisms: (1) AGE accumulation leads to the production of stress stimulants, (2) AGEs directly promote melanin production, and (3) AGEs disrupt cellular epidermal turnover and retard melanin excretion. Among these mechanisms, the third seems most likely as a cause of dull skin and partly a cause of freckles, due to AGEs causing irregular retention of pigment in skin cells.

While investigating potential active compounds in AGE-collagen, we found that CML, CEL, and pentosidine had no effect on melanin production. On the other hand, AGE intermediates (MGO, GO, and 3DG) enhanced melanin production. This indicates that these AGE intermediates are likely melanogenic stimulants. However, these intermediates are also present in collagen-Fru, which had very weak activity. Additionally, the intermediates were less active than whole AGE-collagen itself. This seems to suggest that glycation associated melanin production is influenced by multiple factors to be clarified in future.

Conclusion

This study revealed that AGE-collagen enhances melanin production. While MGO was the most active of the principle components, the various compounds in AGE-collagen work synergistically together. Further research is necessary to better understand the mechanistic details of, and the active compounds responsible for, AGE-induced melanogenesis.

Conflict of interest

The authors have no conflict of interest in this study.

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