ORIGINAL ARTICLE

Correlation between age and the secretions of melanocyte-stimulating cytokines in cultured keratinocytes and fibroblasts

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Key words:
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Summary

Background The majority of skin changes associated with ageing are caused by photoageing and reflect cumulative sun exposure. Although the actinic damage plays a major role in skin pigmentation, it is also important to examine the effects of chronological cellular ageing on the pigmentation. The chief cellular components of the skin other than melanocytes are keratinocytes and fibroblasts, and the influences of age-related changes in those cells on skin pigmentation have not been elucidated.

Objective To clarify the effects of cellular ageing of keratinocytes and fibroblasts on age-related skin pigmentation.

Methods Using ELISA analysis, we measured the level of melanogenic cytokines secreted by cultured keratinocytes and fibroblasts derived from skin of various chronological ages. We also compared the cytokine secretion by cultured keratinocytes between the second and fifth cultures.

Results There was no correlation between age and hepatocyte growth factor (HGF), stem cell factor (SCF), and basic fibroblast growth factor (bFGF) secretion by fibroblasts. On the other hand, a significant positive correlation existed between age and interleukin ((IL)-1α secretion ($R^2 = 0{\cdot}50$, $P = 0{\cdot}002$), and a relatively weak correlation existed between age and endothelin-1 (ET-1) secretion ($R^2 = 0{\cdot}17$, $P = 0{\cdot}051$, not significant). The IL-1α secretion by keratinocytes was significantly increased in the fifth cultures compared with the second cultures ($P < 0{\cdot}005$).

Conclusions These findings suggest that IL-1α secretion increases as cells grow older, and the increased secretion of IL-1α by aged keratinocytes may stimulate HGF production in dermal fibroblasts paracrinely and ET-1 production in keratinocytes autocrinely, which stimulates melanocyte proliferation and induces an increase of tyrosinase activity in melanocytes. Because IL-1α is a primary mediator that responds to inflammation and injury, the transcription of genes involved in skin inflammation may be persistently induced in the aged skin. Thus the increased secretion of IL-1α by aged keratinocytes in the aged skin may play a role in the accentuated cutaneous pigmentation and other skin ageing.

Introduction

The majority of skin changes associated with ageing, such as wrinkles and solar lentigines, are caused by photoageing, and reflect cumulative sun exposure.[1] Although the actinic damage plays a major role in skin pigmentation, it is also important to examine the effects of chronologic cellular ageing. The chief cellular components of the skin other than melanocytes are keratinocytes and fibroblasts, and the influences of age-related changes in those cells on skin pigmentation have not been elucidated.

It has been reported that paracrine linkage between keratinocytes, fibroblasts and melanocytes within the skin plays an important role in epidermal melanization. In response to various stimuli, human keratinocytes secrete various cytokines that are known as mitogen or melanogen for human melanocytes, including endothelin-1 (ET-1)[2–4] and granulocyte-macrophage–colony-stimulating factor (GM-CSF).[5] These cytokines are

suggested to be intrinsic melanogenic ones in ultraviolet (UV) B- and UVA-induced melanosis,[4,5] whereas interleukin (IL)-1α, a proinflammatory cytokine, stimulates the production of ET-1 by keratinocytes and production of hepatocyte growth factor (HGF) by fibroblasts.[3,6] Although HGF, stem cell factor (SCF), and basic fibroblast growth factor (bFGF) have a definite potential to stimulate melanocyte proliferation *in vitro* and *in vivo*,[6–10] keratinocytes do not secrete them at concentrations sufficient to stimulate melanocyte proliferation even after exposure to various stimuli.[11] In contrast to keratinocytes, human fibroblasts secrete several melanogenic cytokines such as bFGF, HGF and SCF,[6,12] which suggests the possibility that the over-expression of these cytokines by dermal fibroblasts may lead to the activation of melanocytes in their overlying epidermis. Based upon this paracrine cytokine network within the skin for epidermal pigmentation mechanisms, it is intriguing to examine the chronological change in cytokine secretion by keratinocytes and fibroblasts. The aim of this study was to elucidate whether keratinocytes and fibroblasts derived from the skin of different chronological ages are different in the potential to produce and secrete several melanogenic cytokines.

Materials and methods

Cell isolation and cell culture

Normal skin specimens were obtained from Japanese patients during plastic surgery. Human fibroblasts were cultured from 19 specimens (age $= 26{\cdot}7 \pm 15{\cdot}6$ years, eight males, 11 females), and keratinocytes were cultured from 16 specimens (age $= 28{\cdot}0 \pm 17{\cdot}1$ years, six males, 10 females). Informed consent was obtained from all patients.

Human keratinocytes were isolated using a modification of the method reported previously.[13,14] Briefly, the skin specimens were washed three times in phosphate-buffered saline (PBS) and finely shredded with scissors and incubated with 0·25% trypsin and 0·02% ethylinediaminetetraacetic acid in PBS for 16–24 h at 4 °C. The epidermis was separated from the dermis with forceps and keratinocytes were isolated from the dermis. Keratinocytes were grown in a modified serum-free keratinocyte growth medium (KGM; Kyokuto Seiyaku, Tokyo, Japan) that consists of MCDB153 with high concentrations of amino acids, transferrin (final concentration 10 $\mu g\ mL^{-1}$), insulin (5 $\mu g\ mL^{-1}$), hydrocortisone (0·5 $\mu g\ mL^{-1}$), phosphorylethanolamine (14·1 $\mu g\ mL^{-1}$) and bovine pituitary extract (40 $\mu g\ mL^{-1}$). The final concentration of Ca^{2+} in the medium was 0·03 mM. Human fibroblasts were isolated for explant after they were separated from the epithelia and grown in FGM (fibroblast growth medium), which consists of Dulbecco's modified Eagle's medium (DMEM), 0·6 mg mL^{-1} glutamine and 10% fetal calf serum (FCS).

Table 1b. Gender differences.

	Male and female n = 19	Male n = 8	Female n = 11
Age (mean ± SD)	26.7 ± 15.6	26.9 ± 18.9	27.1 ± 14.3
HGF (pg mL^{-1})	152 ± 98	128 ± 39	179 ± 126
SCF (pg mL^{-1})	168 ± 69	179 ± 69	161 ± 75
bFGF (pg mL^{-1})	2.6 ± 2	3.2 ± 2.2	2.2 ± 1.8

No gender differences in donor age and cytokine concertrations were found. HGF, Hepatocyte growth factor; SCF, stem cell factor; bFGF, basic fibroblast growth factor.

Table 1a. The age and sex of the 19 hosts, donor region of the skin specimens, and the ELISA values.

Donor	Gender	Age	HGF (pg mL^{-1})	SCF (pg mL^{-1})	bFGF (pg mL^{-1})
Face	F	65	99 ± 14	145 ± 2	1.3 ± 0.2
Buttock	M	58	119 ± 10	223 ± 8	3.8 ± 1.9
Back	M	48	82 ± 6	69 ± 3	2.7 ± 0.1
Chest	F	38	533 ± 25	217 ± 7	5.6 ± 2.4
Abdomen	M	38	103 ± 3	115 ± 8	1.3 ± 0.3
Abdomen	F	29	132 ± 25	296 ± 11	5.4 ± 3.1
Abdomen	F	27	72 ± 1	89 ± 14	2.6 ± 1.6
Upper arm	F	25	175 ± 38	100 ± 4	1.2 ± 0.1
Back	F	23	160 ± 20	83 ± 3	0.6 ± 0.2
Face	F	22	80 ± 6	122 ± 8	1.1 ± 0.2
Upper arm	F	22	232 ± 21	160 ± 7	0.9 ± 0.4
Abdomen	M	21	186 ± 14	217 ± 6	1.7 ± 1.4
Upper arm	M	21	169 ± 42	168 ± 5	5.2 ± 2.1
Lower leg	F	20	160 ± 2	158 ± 11	2.5 ± 1.2
Buttock	F	15	182 ± 40	287 ± 15	0.8 ± 0.1
Face	F	12	148 ± 13	121 ± 12	1.9 ± 0.8
Abdomen	M	11	156 ± 17	202 ± 9	0.9 ± 0.2
Abdomen	M	11	83 ± 5	145 ± 2	2.5 ± 2
Face	M	7	128 ± 36	289 ± 10	7.4 ± 0.8
Mean ± SD		26.7 ± 15.6	152 ± 98	168 ± 69	2.6 ± 2

HGF, Hepatocyte growth factor; SCF, stem cell factor; bFGF, basic fibroblast growth factor; SD, standard deviation.

Measurement of cytokines by ELISA

Cytokine secretion by the cultured keratinocytes and fibroblasts was measured.

Cytokine secretion into fibroblast-conditioned medium.

Third cultures of fibroblasts were used for experiments. Human fibroblasts were seeded in a 60-mm culture dish (three dishes per specimen) at a density of 5×10^5 cells per 5 mL and cultured in FGM. After human fibroblasts had been cultured for 96 h at 37 °C under a 5% CO_2 atmosphere, the medium was collected, and 0·05 mL was used to quantify HGF, 0·1 mL for SCF, and 0·2 mL was used for measurement of bFGF by ELISA.

Cytokine secretion into keratinocyte-conditioned medium.

Second cultures of keratinocytes derived from all 16 donors were used for the first experiments. For ELISA assay, human keratinocytes were seeded in a 60-mm culture dish (three dishes per specimen) at a density of $1{\cdot}5 \times 10^5$ cells per 5 mL and cultured in KGM supplemented with 0·5% FCS. After these human keratinocytes had been cultured at 37 °C under a 5% CO_2 atmosphere for 72 h, the keratinocyte-conditioned medium was collected and quantified for 0·1 mL well^{-1} for IL-1α, ET-1, and 0·1 mL well^{-1} for GM-CSF, respectively, for ELISA.

For the second experiments, the fifth cultures of keratinocytes derived from nine donors, who were less than 30 years old, were used. Cell cultures and ELISA assay were performed in the same way as in the first experiments. The keratinocyte-conditioned medium was collected and the concentrations of IL-1α and ET-1 were quantified.

The ET-1 ELISA kit was purchased from Immuno-Biological Laboratories (Gunma, Japan), and other ELISA kits were purchased from TECHNE Corporation (Minneapolis, MN, U.S.A.). These ELISA kits employ a solid-phase enzyme immunoassay using the multiple-antibody sandwich principle. A human purified polyclonal antibody specific for human ET-1, SCF, HGF, bFGF and human purified monoclonal antibodies specific for IL-1α and GM-CSF were attached to 96-well microtitre plates. Levels of immunoreactive cytokines were measured as A490 by ELISA plate reader (Microplate Reader Model 550; Bio-Rad Laboratories, Hercules, CA, U.S.A.). Cytokine levels in the culture samples were determined by comparing their absorbance with that produced by standards. Standard curves were linear from 3·9 to 125 pg mL^{-1} for IL-1α derivatives, from 3·13 to 100 pg mL^{-1} for ET derivatives, from 3·9 to 250 pg mL^{-1} for GM-CSF derivatives, from 62·5 to 4000 pg mL^{-1} for HGF derivatives, from 31·2 to 2000 pg mL^{-1} for SCF derivatives and from 0·5 to 64 pg mL^{-1} for bFGF derivatives.

In the first experiments, a comparison of the cytokine concentration between male and female was carried out using the unpaired t-test. Scatter diagrams showing the relationship between age and cytokine concentration were

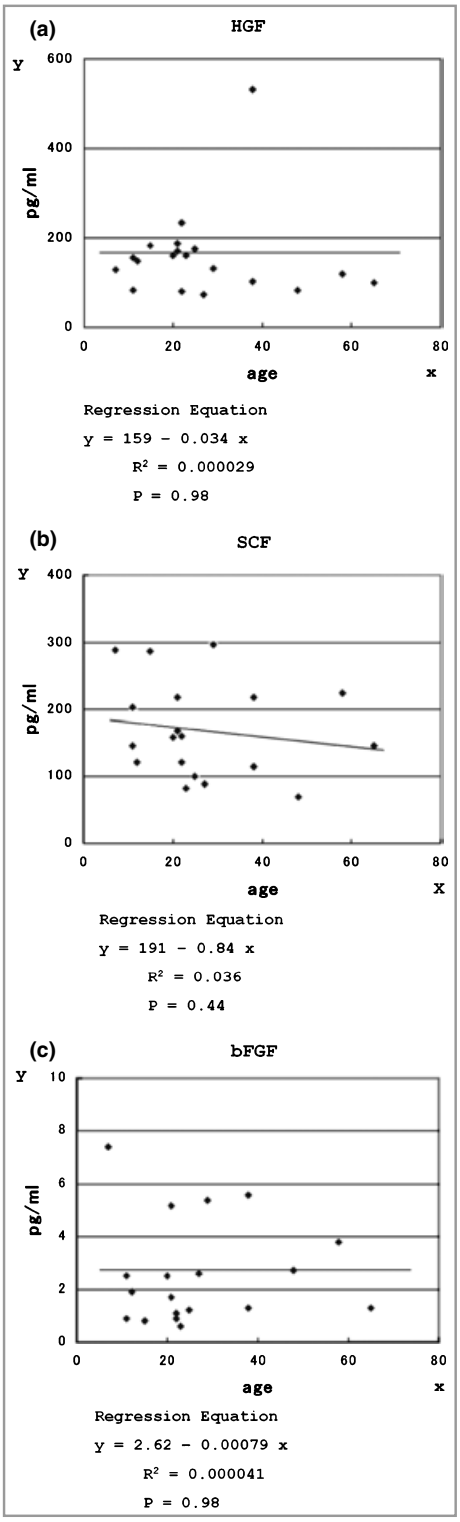

Figure 1. Scatter diagram showing the relationship between donor age and value of cytokine concentration in fibroblasts. The lines represent the linear regression equation. N = 19, R, coefficient of determination. (a) Hepatocyte growth factor (HGF), y = 159–0·034x, R^2 = 0·000029, P = 0·98. (b) Stem cell factor (SCF), y = 191–0·84x, R^2 = 0·036, P = 0·44. (c) Basic fibroblast growth factor (bFGF), y = 2·62–0·00079x, R^2 = 0·000041, P = 0·98.

Table 2a. The age and sex of the 16 hosts, donor region of the skin preparations, and the ELISA values.

Donor site	Gender	Age	IL-1α (pg mL^{-1})	ET-1 (pg mL^{-1})	GM-CSF (pg mL^{-1})
Abdomen	F	64	21.3 ± 6.7	26.5 ± 8	20.2 ± 2.8
Buttock	M	58	27.4 ± 1.7	22.3 ± 2	30.9 ± 4.1
Back	M	48	28.1 ± 5.1	27.2 ± 3.8	37.4 ± 1.2
Abdomen	M	38	11.1 ± 0.6	19.9 ± 1.6	11.7 ± 1.4
Chest	F	38	23.5 ± 4.3	22.4 ± 0.7	10 ± 1.1
Abdomen	F	29	15.3 ± 6.8	24.8 ± 1.3	26.8 ± 3.1
Abdomen	F	27	23.5 ± 5.6	30.9 ± 5.6	19.7 ± 2.6
Abdomen	F	26	4.5 ± 0.5	9.5 ± 2.7	7.9 ± 0.5
Upper arm	F	25	9.8 ± 3.8	5.1 ± 0.2	28.9 ± 0.5
Upper arm	F	20	4.2 ± 0.8	4.6 ± 0.1	21.6 ± 3.8
Upper arm	F	19	6.2 ± 1.2	6.3 ± 0.1	10.3 ± 2.8
Buttock	F	15	10.1 ± 1.9	18.8 ± 0.8	30.4 ± 1.3
Face	F	12	5.4 ± 0.2	6.1 ± 0.5	13 ± 0.7
Abdomen	M	11	19.2 ± 6	19.8 ± 3.1	11.5 ± 1.9
Abdomen	M	11	7.3 ± 0.8	22.8 ± 0.9	22 ± 1.7
Face	M	7	6.3 ± 0.8	16.1 ± 0.9	16.8 ± 3.2
Mean ± SD		28 ± 17.1	14 ± 8.6	17.7 ± 8.7	19.9 ± 9

IL-1α, Interleukin-1α; ET-1, endothelin-1; GM-CSF, granulocyte-macrophage–colony-stimulating factor; SD, standard deviation.

Table 2b. Gender differences.

	Male and female n = 16	Male n = 6	Female n = 10
Age (mean ± SD)	28 ± 17.3	28.3 ± 22	27.8 ± 14.5
IL-1α (pg mL^{-1})	14 ± 8.6	16.6 ± 9.8	12.4 ± 7.9
ET-1 (pg mL^{-1})	17.7 ± 8.7	21.4 ± 3.7	15.5 ± 10.2
GM-CSF (pg mL^{-1})	19.9 ± 9	21.7 ± 10.6	18.9 ± 8.3

No gender differences in donor age and cytokine concertrations were found. IL-1α, Interleukin-1α; ET-1, endothelin-1; GM-CSF, granulocyte-macrophage–colony-stimulating factor; SD, standard deviation.

drawn, simple linear regression equations were calculated and the simple linear regression test was used to determine whether there was any correlation between age and concentration of cytokine. $P < 0{\cdot}05$ was considered statistically significant.

In the second experiments, a comparison of the cytokine concentrations between the second and fifth culture keratinocytes was carried out using paired t-test. $P < 0{\cdot}05$ was considered statistically significant.

Results

Measurement of cytokines by ELISA

Cytokine secretion into fibroblast-conditioned medium.

The age and sex of the 19 hosts, donor region of the skin specimens and the ELISA values are shown (Table 1a). No gender differences in donor age and cytokine (HGF, SCF, bFGF) concentration were found between males and females (Table 1b). There was no correlation between age and cytokine concentration (R: coefficient of determination, HGF: $R^2 = 0{\cdot}000029$; SCF: $R^2 = 0{\cdot}036$; bFGF: $R^2 = 0{\cdot}000041$) (Fig. 1a–c).

Cytokine secretion into keratinocyte-conditioned medium.

The age and sex of the 16 hosts, donor region of the skin specimen, and the ELISA values are shown (Table 2a). No gender differences in the donor age and cytokine (IL-1α, ET-1, GM-CSF) concentration were found between males and females (Table 2b).

There was a significant correlation between age and IL-1α concentration ($R^2 = 0{\cdot}50$, $P = 0{\cdot}002$). There was a relatively weak correlation between age and ET-1 concentration, but the correlation was not significant ($R^2 = 0{\cdot}17$, $P = 0{\cdot}051$). No correlation existed between age and GM-CSF concentration ($R^2 = 0{\cdot}10$, $P = 0{\cdot}23$) (Fig. 2a–c).

ELISA of IL-1α and ET-1 released into the keratinocyte-conditioned medium revealed that the concentration of IL-1α was significantly increased in fifth cultures (mean ± SD = 17·3 ± 6·2 pg mL^{-1}, $P < 0{\cdot}005$) compared with second cultures (mean ± SD = 7·7 ± 3·5 pg mL^{-1}) (Fig. 3a). The levels of ET-1 did not differ significantly between fifth (mean ± SD = 13·8 ± 6·1 pg mL^{-1}) and second cultures (mean ± SD = 12·7 ± 8 pg mL^{-1}) (Fig. 3b).

Discussion

The results of the present study suggest that a statistically significant positive correlation exists between age and IL-1α secretion by cultured keratinocytes. There was a weak correla-

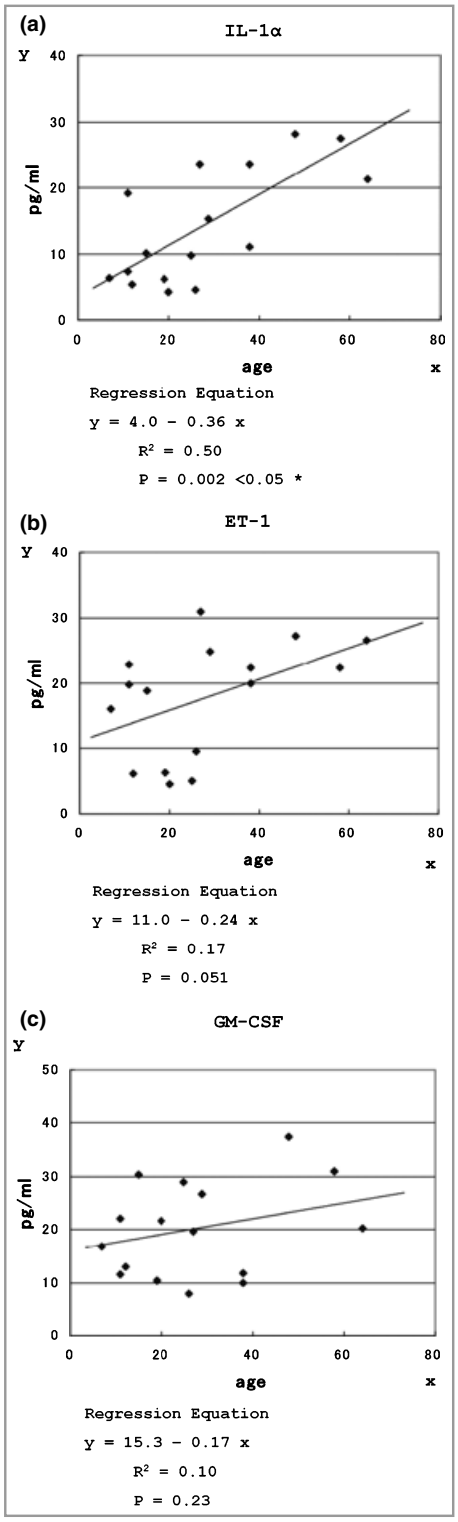

Figure 2. Scatter diagram showing the relationship between donor age and value of cytokine concentration in keratinocytes. The lines represent the linear regression equation. N = 16, R, coefficient of determination. (a) Interleukin (IL)-1α, y = 4·0 + 0·36x, R^2 = 0·50, P = 0·002 < 0·05. (b) Endothelin-1 (ET-1), y = 11·0 + 0·24x, R^2 = 0·17, P = 0·051. (c) Granulocyte-macrophage–colony-stimulating factor (GM-CSF), y = 15·3 + 0·17x, R^2 = 0·10, P = 0·23.

tion between age and ET-1 secretion by cultured keratinocytes, but the correlation was not significant. After these results were obtained, we next compared the secretion of IL-1α and ET-1 by keratinocytes between old (fifth) and young (second) cultures. It was revealed that the level of IL-1α was significantly increased in the old cultures, but levels of ET-1 did not differ significantly compared with the young cultures. It was shown that IL-1α stimulated ET-1 production by keratinocytes[3] and that ET-1 induced an increase in tyrosinase activity and stimulated melanocyte proliferation.[4] Consistently, there was a significant correlation between IL-1α and ET-1 secretion by keratinocytes in culture for 3 days (y = 6·8 + 0·78x, R^2 = 0·59, P = 0·0005)(Fig. 4). In our study, there was a significant correlation between age and IL-1α secretion by keratinocytes. On the other hand, the correlation between age and ET-1 secretion was not significant, although a weak correlation existed. One possible explanation for these findings is that the autocrine stimulation by IL-1α might not have worked on the aged keratinocytes sufficiently under our culture conditions. This is supported by our second experiment; the levels of ET-1 secretion did not differ significantly, although IL-1α secretion was significantly increased in old (the fifth) cultures compared with young (the second) cultures. In the second experiment, only young cells derived from nine donors who were younger than 30 years were used, because keratinocytes derived from aged skin (> 30 years of age) can no longer proliferate vigorously after three to four subcultures. In our preliminary study, the keratinocytes derived from a 1-year-old child could be cultivated serially for about seven to eight passages under the culture conditions described in the above section on Cell isolation and cell culture, when they are subcultured every 7 days. At the 7th or 8th cultures, most cultures were composed of enlarged and flattened cells, and such cultures were considered senescent, and subculturing was discontinued. These findings were consistent with those reported by Boyce and Ham,[15] and the chronological cellular age of the 5th culture can be estimated as 30–40 years older than that of the 2nd culture.

It was reported that ET-1 production by keratinocytes increased after irradiation with UVB accompanied by significant secretion of IL-1α,[3] which suggests that IL-1α secreted by keratinocytes plays the role of trigger in the UVB hyperpigmentation in the epidermis. Our present study suggests that older keratinocytes may accentuate the cellular activities of melanocytes by secreting more IL-1α than young keratinocytes. Possibly, in the aged skin, melanocytes are stimulated persistently by keratinocytes as if the skin were irradiated with UVB. As a result, epidermal pigmentation might be accentuated in the aged skin. As for cases where epidermal keratinocytes produce and secrete a larger amount of ET-1, leading to an accentuated epidermal pigmentation, it has been reported that such a mechanism occurs *in vivo* in lentigo senilis.[16]

In the present study, there was no significant correlation between age and cytokine secretion by cultured fibroblasts. It was reported that human dermal fibroblasts from aged

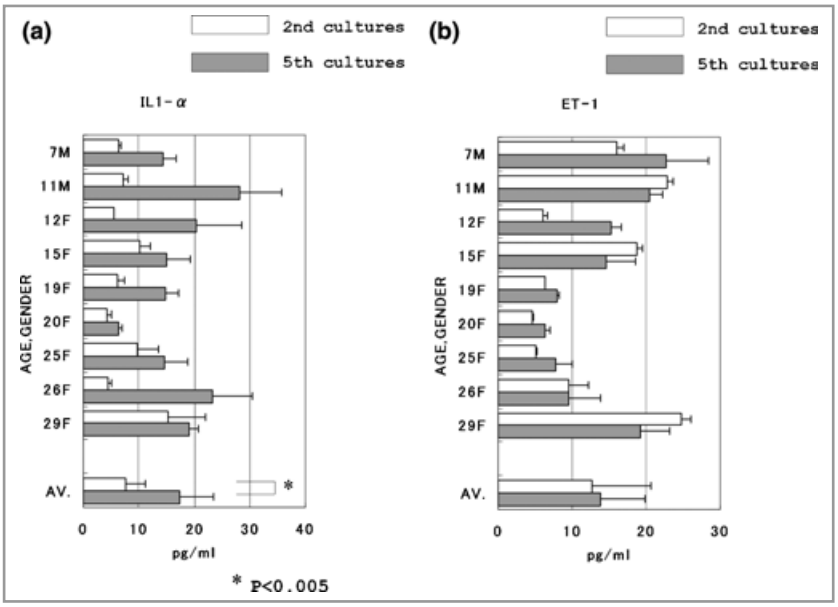

Figure 3. Comparison of interleukin (IL)-1α and endothelin-1 (ET-1) secretion by keratinocytes between second cultures (□) and fifth cultures (■). Each ELISA value (values are means ± SD derived from three wells of each specimen) of nine donors and average value (AV) are shown. The lowest: comparison of the average value between two groups. *P < 0·005 to second cultures. (a) IL-1α. (b) ET-1.

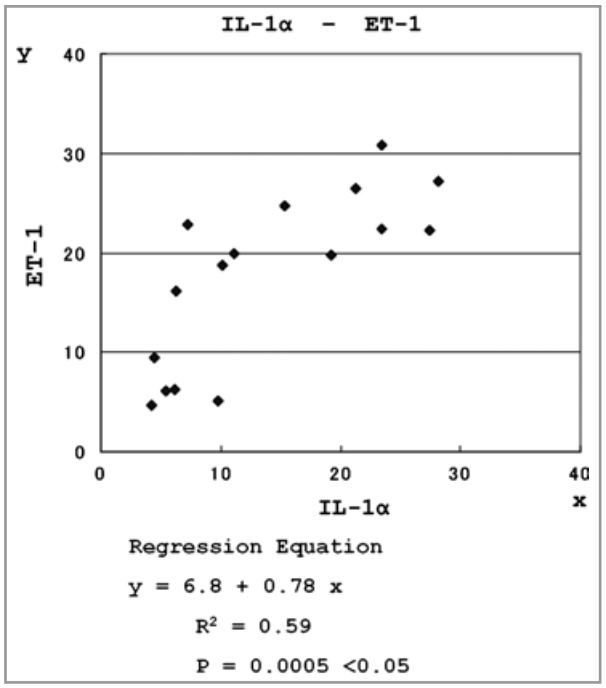

Figure 4. Scatter diagram showing the relationship between interleukin (IL)-1α and endothelin-1 (ET-1) concentrations in keratinocytes. The lines represent the linear regression equation. N = 9, R, coefficient of determination. $y = 6{\cdot}8 + 0{\cdot}78x$, $R^2 = 0{\cdot}59$, $P = 0{\cdot}0005 < 0{\cdot}05$*.

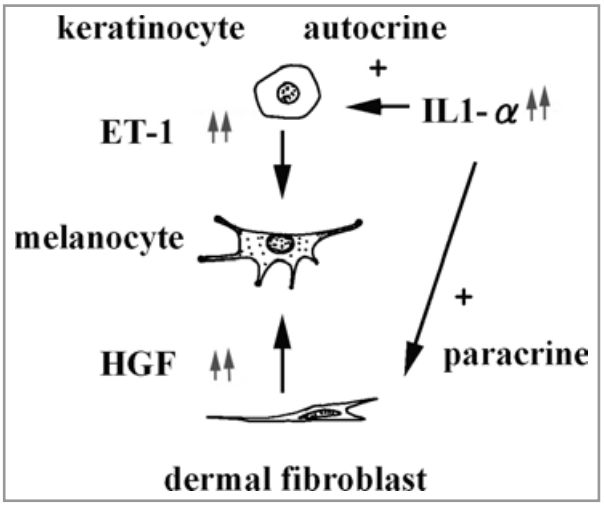

Figure 5. Schematic showing the hypothesis of the accentuated pigmentation of the aged skin. Increased secretion of interleukin (IL)-1α by the keratinocytes in the aged skin stimulates hepatocyte growth factor (HGF) production by fibroblasts paracrinely and endothelin-1 (ET-1) production by keratinocytes autocrinely, which stimulate melanocyte proliferation and induce an increase of tyrosinase activity in melanocytes.

donors, >80 years, produced more HGF than fibroblasts from young and middle-aged donors, and the production of HGF by human embryonic lung fibroblasts increased sharply after about 70% completion of their lifespan in culture.[17] In our study, there was no correlation between age and HGF secretion, probably because the age of donor ranged from 7 to 65. It is presumed that the production of HGF by human cultured fibroblast would increase abruptly from the age about 70–80.

IL-1α was reported to stimulate the expression of HGF mRNA and protein production in cultured fibroblasts.[18,19] It is probable that the production of HGF by fibroblasts in the aged skin is increased due to the paracrine stimulation by IL-1α secreted by aged keratinocytes, and consequently melanocytes are stimulated by HGF. Our results support the hypothesis that increased IL-1α secreted by the aged keratinocytes stimulates HGF production by dermal fibroblasts paracrinely and ET-1 production by keratinocytes autocrinely, and these cytokines

stimulate melanocyte proliferation and induce an increase of tyrosinase activity in melanocytes (Fig. 5). Furthermore, because IL-1α is a primary mediator that responds to inflammation and injury,[20] the transcription of genes involved in skin inflammation may be persistently induced in the aged skin. Thus, it is probable that the aged skin is always exposed to conditions such as inflammation, and the increased potential to secret IL-1α in aged keratinocytes is associated with various kinds of skin ageing in addition to epidermal pigmentation.

One of the limitations of this study is that skin specimens were obtained from various sites of the body; site-specific differences of cytokine secretion cannot be assessed because of the small number of specimens involved. It is an essential variable whether the specimen was obtained from a sun-exposed or sun-protected site. If the expression of IL-1α mRNA by keratinocytes was measured in fresh skin, it is probable that the expression was elevated in the sun-exposed skin compared with sun-protected skin, and it is possible that the expression of ET-1 mRNA by keratinocyte and HGF mRNA by fibroblasts was upregulated in the sun-exposed skin. To exclude the influence of acute sunlight exposure of the skin on cytokine secretion, we used the isolated skin cells in culture (no use of freshly isolated cells). Actually, the concentration of IL-1α secreted by keratinocytes derived from sun-exposed skin [face, $n = 2$, ages $= 7$ and 12; concentration $= 5{\cdot}9 \pm 0{\cdot}6$ pg mL^{-1} (mean ± SD)] is not more than that derived from sun-protected skin [abdomen and buttock, $n = 3$, ages $= 11$, 11 and 15 (under 20); concentration $= 12{\cdot}2 \pm 6{\cdot}2$ (mean ± SD)]. Because cultured cells were used to measure cytokine secretion, it is inevitable that biological age of the cells used in our study was greater than that of donors. To overcome this limitation, the expression of cytokine mRNA in fresh skin should now be studied, dividing specimens into two groups: skin derived from a sun-exposed site and from a sun-protected site.

In conclusion, our findings suggest that the secretion of IL-1α by human keratinocytes increases as cells age, and the increased secretion of IL-1α by keratinocytes in the aged skin may play a role in the accentuated cutaneous pigmentation and skin ageing.

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