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ORIGINAL ARTICLE

Epidermal hyperpigmentation in non-syndromic solitary cafe-au-lait macules may be associated with increased secretion of endothelin-1 by lesional keratinocytes

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Abstract

To clarify the mechanism of accentuated melanisation in non-syndromic solitary cafe-au-lait macules we used an enzyme-linked immunosorbent assay (ELISA) to measure the concentration of melanogenic cytokines secreted by cultured keratinocytes and fibroblasts derived from the skins of the macules and compared them with those derived from normal people. Endothelin-1 (ET-1) was significantly increased in cultured keratinocytes in the macules compared with the normals. In contrast, the secretion of other cytokines secreted by keratinocytes or fibroblasts did not differ between the groups. It may be that the increased secretion of ET-1 by epidermal keratinocytes has a role in the accentuated epidermal melanisation seen in non-syndromic macules.

Key Words: *Solitary café-au-lait macule, melanogenic cytokine, endothelin, melanocyte, keratinocyte, fibroblast, ELISA (enzyme-linked immunosorbent assay)*

Introduction

The cafe-au-lait macule is a non-hairy, light to dark brown, well-circumscribed cutaneous macular area. Such macules are generally divided into two groups: multiple (which are part of the cutaneous signs in patients with syndromic diseases such as neurofibromatosis type-1 and Albright syndrome), and solitary macules (which develop in normal people and are not associated with syndromic diseases) [1]. Histological studies of solitary macules have shown increased epidermal melanisation and a normal number of melanocytes with raised tyrosinase activity, while those of multiple macules have shown increased epidermal melanisation and increased number of melanocytes and a normal amount of tyrosinase activity [2–5].

It has recently been reported that paracrine linkage between keratinocytes, fibroblasts, and melanocytes within the skin have an important role in accentuated epidermal melanisation. In response to various stimuli, the keratinocytes secrete various cytokines that are known as mitogen or melanogen for the melanocytes including endothelin-1 (ET-1) [6–8] and granulocyte macrophage colony stimulating factor (GM-CSF) [9]. Although hepatocyte growth factor (HGF), stem cell factor (SCF), and basic fibroblast growth factor (bFGF) have definite potential for stimulating melanocyte proliferation in vitro and in vivo [10–13], keratinocytes do not secrete them at concentrations sufficient to stimulate proliferation of melanocytes even after exposure to various stimuli [14]. In contrast to keratinocytes, human fibroblasts secrete several melanogenic cytokines such as bFGF, HGF, and SCF under rapidly growing conditions or during inflammation [15,16], which suggests a possibility that their over-expression may lead to the activation of melanocytes in their overlying epidermis.

We have reported previously [17] that the epidermal hyperpigmentation in multiple macules of neurofibromatosis type 1 might be associated with

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increased dermal fibroblasts-derived SCF and HGF. In the present study, we elucidated whether keratinocytes or fibroblasts localised in solitary macules differ in their potential to secrete melanogenic cytokines including ET-1, GM-CSF, HGF, SCF, and bFGF compared with normal skin.

Materials and methods

Cell isolation and cell culture

Specimens of solitary macules were obtained from six Japanese patients, five of whom were female, age range 11–26, mean (SD) 19 (6) years (Table I). Those who were thought to have syndromic café-au-lait (neurofibromatosis, Albright syndrome) were excluded from the study. Patients with Becker naevus were also excluded. Keratinocytes and fibroblasts were cultured from all six patients. Specimens of normal skin were obtained from 18 Japanese patients during plastic surgery. Keratinocytes were cultured in 11 specimens, seven of whom were female, age range 7–29, mean (SD) 18 (8) years, and fibroblasts were cultured in 14 specimens, nine of whom were female, age range 7–29, mean (SD) 19 (7) years. Informed consent was obtained from all patients before operation.

Human keratinocytes were isolated using the method reported previously [17]. Briefly, the specimens were washed three times in phosphate buffered saline (PBS) and finely shredded with scissors, and incubated with 0.25% trypsin and 0.02% EDTA in PBS for 16–24 hours at 4°C. The epithelium was separated from the dermis with forceps, and keratinocytes were isolated from the subepithelial side. Keratinocytes were grown in a modified serum-free keratinocyte growth medium (KGM; Kyokuto Seiyaku, Tokyo), which consists of MCDB153 with high concentrations of amino acids, transferrin (final concentration 10 μg/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), phosphorylethanolamine (14.1 μg/ml), and bovine pituitary extract (40 μg/ml). The final concentration of calcium ions in the medium was 0.03 mmol. Human fibroblasts were isolated for explant after they had been separated from the epithelium, and grown in fibroblast growth medium (FGM), which consists of Dulbecco's modified Eagle's medium (DMEM), 0.6 mg/ml glutamine, and 10% fetal calf serum (FCS).

Table I. The profiles of six patients and the donor sites from which the macules were taken.

Case No.	Age (years)	Sex	Region
1	11	F	Lower leg
2	15	F	Thigh
3	16	F	Thigh
4	23	F	Elbow
5	25	M	Back
6	26	F	Lower leg
(Age mean (SD) 19.3 (6.1) years)			

Experiment 1 (measurement of cytokines by enzyme-linked immunosorbent assay (ELISA)

The secretion of cytokines by the cultured keratinocytes and fibroblasts was compared between the two groups to find out which cytokines may be involved in the activation of melanocytes in solitary macules.

Cytokine secretion into keratinocyte-conditioned medium

The second passage of keratinocyte cultures was used for the experiments. For the ELISA, human keratinocytes were seeded in 60 mm culture dish (three dishes/specimen) at a density of 1.5×10^5 cells/5 ml, and cultured in KGM supplemented with 0.5% FCS. After they had been cultured at 37°C in a 5% carbon dioxide atmosphere for 72 hours, the keratinocyte-conditioned medium was collected and 0.1 ml/well was used to quantify ET-1, and 0.1 ml/well was used to quantify GM-CSF for measurement by ELISA.

Cytokine secretion into fibroblast-conditioned medium

The third passage of fibroblast cultures was used for the experiments. Human fibroblasts were seeded in 60 mm culture dish (three dishes/specimen) at a density of 5×10^5 cells/5 ml and cultured in FGM. After human fibroblasts had been cultured for 96 hours at 37°C in a 5% carbon dioxide atmosphere, the medium was collected, and 0.05 ml was used for HGF, 0.1 ml for SCF, and 0.2 ml for bFGF was used for ELISA.

The ET-1 ELISA kit was bought from Immuno-Biological Laboratories (Gunma, Japan), and other ELISA kits from TECHNE Corporation (Minneapolis, USA). These ELISA kits use 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 GM-CSF were attached to 96-well microtitre plates. Concentrations of immunoreactive cytokine were measured as A490 by an ELISA plate reader (MICROPLATE READER Model 550; Bio-Rad Laboratories). Cytokine concentrations in samples of culture were calculated by comparing their absorbance with that produced by standards. The standard curve was linear from 3.13 to 100 pg/ml for ET derivatives, from 3.9 to 250 pg/ml for GM-CSF

derivatives, from 62.5 to 4000 pg/ml for HGF derivatives, from 31.2 to 2000 pg/ml for SCF derivatives, and from 0.5 to 64 pg/ml for bFGF derivatives. The concentrations were compared using Student's *t*-test.

Experiment 2 (measurement of ET-1 by ELISA, second passage compared with fifth passage)

ET-1 secretion into keratinocyte-conditioned medium. The increased secretion of ET-1 in the macule group compared with normals had been studied in experiment 1, so next, ET-1 secretion by the fifth passage of keratinocyte cultures was studied to elucidate whether the increased ET-1 secretion in macule-derived keratinocytes might also be seen after further three subcultures, and whether the further subcultures might influence the ET-1 secretion in the two groups. The keratinocyte lines derived from six patients with solitary macules and those derived from 9 of 11 normal skin specimens (seven of whom were females, age range 7–29, mean (SD) 18 (8) years) were used for the experiment. The measurement of the concentration of ET-1 by ELISA was made in the same way as in experiment 1. The concentrations in the two groups were compared using Student's *t*-tests.

Results

Experiment 1

Cytokine secretion into keratinocyte-conditioned medium (Table IIa). ELISA of factors released into the keratinocyte-conditioned medium showed significantly higher concentrations of ET-1 in the macules than in normals (Figure 1*a*). The levels of GM-CSF did not differ significantly between the two groups (Figure 1*b*).

Table II. (a) Mean (SD) concentration of cytokines in keratinocyte-conditioned medium measured by ELISA. (b) Mean (SD) concentration of cytokines in fibroblast-conditioned medium measured by ELISA.

(a)

Cytokines	Macules ($n=6$)	Normal ($n=11$)
ET-1 (pg/ml)	42.1 (17.6)	15.0 (9.2)
GM-CSF (pg/ml)	25.6 (10.0)	19.0 (7.8)

(b)

Cytokines	Macules ($n=6$)	Normal ($n=14$)
HGF (pg/ml)	196 (78)	147 (45)
SCF (pg/ml)	228 (45)	174 (74)
bFGF (pg/ml)	1.7 (2.1)	2.5 (2.1)

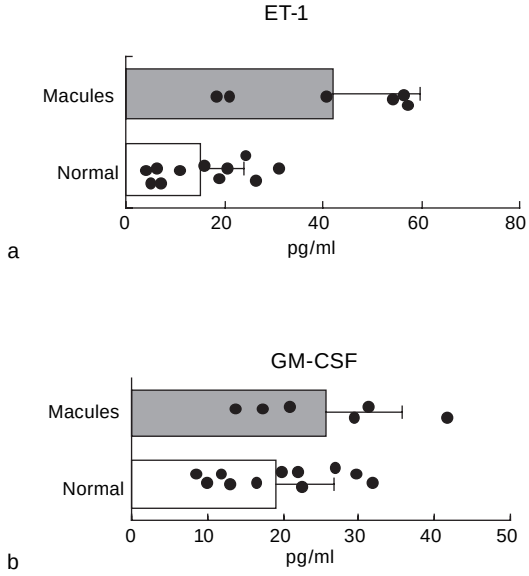

Figure 1. Mean (SD) secretion of cytokines by keratinocytes for 3 days in culture (macules $n=6$, normals $n=11$). (*a*) ET-1 ($p < 0.01$), (*b*) GM-CSF.

Cytokine secretion into fibroblast-conditioned medium (Table IIb). ELISA of factors released into the fibroblastic cell-conditioned medium showed no significant differences between the two groups in concentrations of HGF, SCF, and bFGF (Figure 2*a*–*c*).

Experiment 2

ET-1 secretion into keratinocyte-conditioned medium (second passage compared with fifth passage) (Table III). ELISA showed significantly higher concentrations of ET-1 in the macule group than among normals (Figure 3). Comparison between second and fifth passage of keratinocyte cultures in the macule group showed that the concentration of ET-1 after the fifth passage was lower than after the second passage, but the difference was not significant. Comparison between the normal groups showed no significant differences in concentrations of ET-1.

Discussion

Because solitary macules are considered a congenital skin disease, we thought that their mechanism of hyperpigmentation could be attributed not to exogenous stimulation but to the lesional cells themselves. Although there is a possibility that the

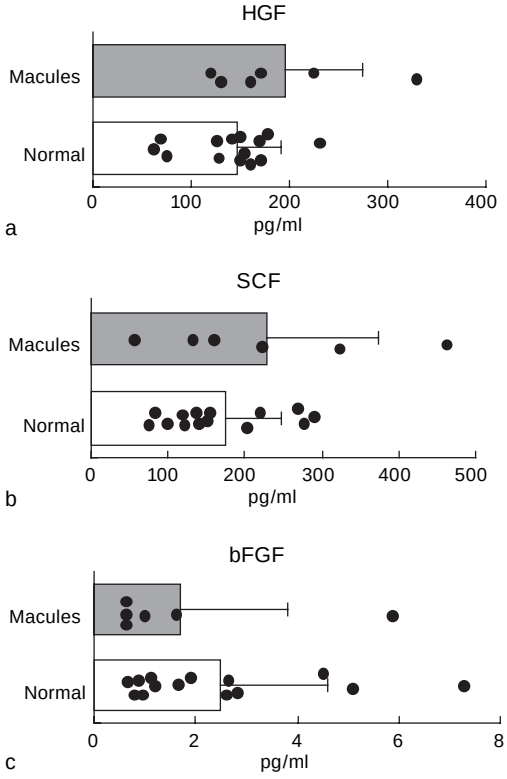

Figure 2. Mean (SD) secretion of cytokines by fibroblasts for 4 days in culture (macules $n=6$, normals $n=14$). (*a*) HGF, (*b*) SCF, (*c*) bFGF. There were no significant differences between the groups.

melanocyte itself is responsible for the hyperpigmentation, we first studied the effects of lesional keratinocytes and fibroblasts. We used isolated cells in culture to exclude extrinsic influences such as solar irradiation. If the evaluation of cytokine or mRNA expression had been studied using raw cells or tissues, the extrinsic influences on pigmentation could not be excluded. In addition, using isolated cells in culture, the influence of interactions among keratinocytes, fibroblasts, and melanocytes was excluded, and intrinsic property of cells could be evaluated. However, we might have altered the properties of the culture or subcultures. "Early passage" cells were used to minimise the influence of the cultures or subcultures. We extended our study to experiment 2 to investigate the influence of subcultures on alterations of cellular property.

Histological studies of solitary macules showed increased epidermal melanisation and normal number of melanocytes with increased tyrosinase activity, while those of multiple macules in neurofibromatosis type 1 showed increased epidermal melanisation and an increased number of melanocytes with normal tyrosinase activity [2–5]. We previously reported that the fibroblasts located in multiple macules in neurofibromatosis type 1 secreted more SCF and

Table III. The concentration of ET-1 in keratinocyte-conditioned medium measured by ELISA (keratinocyte; second passage and fifth passage).

(a) Macules

	ET-1 (pg/ml)	
Case No.	Second passage ($n=6$)	Fifth passage ($n=6$)
1	56.0 (2.2)	39.0 (5.1)
2	55.2 (21.5)	33.7 (4.1)
3	58.7 (16.4)	48.1 (11.7)
4	22.1 (4.0)	30.3 (5.4)
5	40.8 (14.6)	37.9 (6.2)
6	19.6 (1.9)	21.2 (1.9)
Mean (SD)	42.1 (17.6)	35.0 (9.1)

(b) Normal skin

	ET-1 (pg/ml)	
Case No.	Second passage ($n=9$)	Fifth passage ($n=9$)
1	16.1 (0.9)	22.7 (5.7)
2	22.8 (0.9)	20.5 (1.7)
4	6.1 (0.5)	15.3 (1.3)
5	18.8 (0.8)	14.6 (4.0)
6	6.3 (0.1)	8.0 (0.3)
7	4.6 (0.1)	6.3 (0.7)
8	5.1 (0.2)	7.7 (2.3)
9	9.5 (2.7)	9.6 (4.2)
11	24.8 (1.3)	19.2 (4.0)
Mean (SD)	12.7 (8.0)	13.8 (6.1)

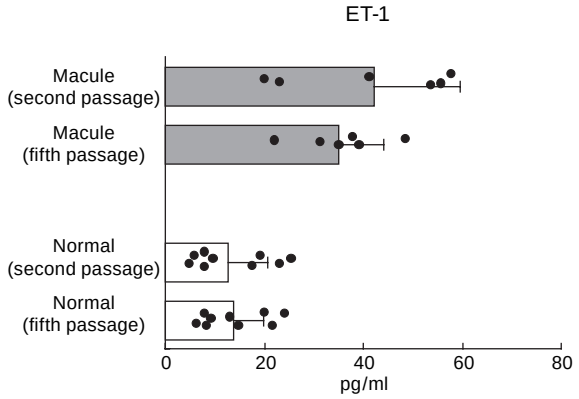

Figure 3. Mean (SD) ET-1 secretion by keratinocytes for 3 days in culture (macule groups $n=6$, normal groups $n=9$). The difference between macules and normals (fifth passage) was significant ($p<0.01$).

HGF than non-lesional fibroblasts [17]. Because HGF and SCF have a good capacity to stimulate melanocyte proliferation [12], we suggested that raised secretion of HGF and SCF by lesional fibroblasts might be associated with epidermal hyperpigmentation in the multiple macules in neurofibromatosis type 1. The histological findings of multiple macules in neurofibromatosis type 1

(increased epidermal melanisation and increased number of melanocytes) are consistent with our previous report [17]. However, the characteristic finding of solitary macules differs from that of the multiple ones in neurofibromatosis type 1 [2–5]. We have therefore hypothesised that the mechanism of hyperpigmentation in solitary macules might differ from that in multiple ones, and so we studied the secretion of melanogenic cytokine by keratinocytes and fibroblasts in the skin of solitary macules, compared with healthy control skin. It is quite difficult to obtain the non-lesional skin from patients with solitary macules although it is the best control, so the specimens of normal skin derived from other normal patients during plastic surgery (chiefly during the correction of dog-ears) were used as controls.

We have shown that the degree of secretion of ET-1 by epidermal keratinocytes located in solitary macules was significantly higher than that secreted by keratinocytes derived from normal skin of normal people. We next studied the degree of secretion of ET-1 during the fifth passage of keratinocyte cultures. The concentration of ET-1 in the macule group was also significantly higher than in the normal group. It supports the hypothesis that the increased secretion of ET-1 by keratinocytes derived from solitary macules might be an intrinsic property. Because ET-1 has the capacity to induce an increase in the tyrosinase activity of melanocytes [7], increased secretion of ET-1 by keratinocytes located in solitary macules stimulates the tyrosinase activity of melanocytes, which may cause the accentuated pigmentation. Our results are consistent with the previous report that solitary macules show increased epidermal melanisation with increased tyrosinase activity [4,5].

In cases 4 and 6, the values of ET-1 concentration in keratinocytes derived from solitary macules were comparatively low (case 4 = 22.1 pg/ml and case 6 = 19.6 pg/ml, respectively). Considering that in 4 of 11 healthy controls the ET-1 concentration was more than 19 pg/ml, the possibility remains that the mechanism of hyperpigmentation of some other solitary macules might differ from what we have suggested here. There may be other possible explanations as to how melanocytes are activated in solitary macules: melanocytes in such skin acquire an increased sensitivity to several melanogenic stimuli and produce more melanin; or the lesional melanocytes themselves acquire a potential to produce more melanin.

In conclusion, we suggest that increased secretion of ET-1 in epidermal keratinocytes from solitary macules have a role in their accentuated epidermal pigmentation, although the mechanism might not be the same for all such lesions.

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