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Fibroblast-conditioned medium significantly affects the morphological and functional differentiation of human epidermal melanocytes, but much less of human melanoma cells.

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ABSTRACT **Our goal is to study the role of dermal fibroblasts in the proliferation and differentiation of epidermal melanocytes using a reconstituted epidermal melanin unit (EMU). In order to achieve this goal, we have investigated the effect of fibroblast-conditioned medium (FCM) on the growth and melanogenesis of normal human epidermal melanocytes (NHEM) from neonatal foreskin using SK-MEL23 human melanoma cells as controls.**

NHEM and SK-MEL23 cells grown on type-I collagen gel alone revealed short dendrites. The culture on type-I collagen gel and fibroblasts resulted in prominent dendricities of NHEM. The FCM, which was likely to contain extracellular matrix (ECM) proteins and cytokines derived from fibroblasts, remarkably enhanced the dendricity of NHEM and their attachment to petri dishes/culture plates, but did so to a lesser degree with control SK-MEL23 cells. However, it did not affect the proliferation of both NHEM and SK-MEL23 cells. The FCM treatment decreased the tyrosinase activity of NHEM, while this decrease was not seen in melanoma cells. These studies suggest that cytokines and ECM proteins from dermal fibroblasts are important in regulating the functional and morphological differentiation of epidermal human melanocytes, and that this biological effect is much smaller in their neoplastic counter parts, melanoma cells.

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Key words: Melanocyte, SK-MEL23, Fibroblast, Collagen

Introduction

Melanin pigmentation is brought about by a symbiotic interaction of melanocytes and surrounding cells in the skin. This symbiotic relationship between a melanocyte and an associated pool of keratinocytes (one to 36 ratio) is called as epidermal melanin unit (EMU)[1]. The concept of EMU has been shown to be quite a useful means to explain the pathophysiology of abnormal melanin pigmentation in the skin, especially for congenital circumscribed hypomelanotic and hypermelanotic skin diseases[2]. However, in many acquired pigmentary diseases, there is an involvement of dermal components including dermal inflammatory cells and fibroblasts. Furthermore, many external stimuli that are known to be potent melanogenic agents, e. g., UV light, affect not only epidermal cells (keratinocytes) but also dermal cells (fibroblasts). Previous studies using the EMU model for the characterization of the abnormal melanin pigmentation mechanism have been largely based on the interaction of melanocytes and keratinocytes.

The cell-cell interaction is carried out by cytokines, hormones, neurotransmitters, and cell membrane adhesion molecules[3]. Keratinocytes and fibroblasts produce many cytokines and extracellular matrix proteins, which can potentially stimulate melanogenesis. Yaar and Gilchrest showed[4] that phor-

bol esters, cyclic adenosine monophosphate (cAMP), UV radiation, keratinocyte-conditioned medium (K-CM), nerve growth factor (NGF), vitamin D_3 (VD_3), and prostaglandin E_2 (PGE_2) could induce the melanogenesis. They also showed that phorbol esters, basic fibroblast growth factor (bFGF), cAMP, α melanocyte stimulating hormone (αMSH), serum, and K-CM could induce proliferation of melanocytes, and that phorbol esters, cAMP, K-CM, UVR, VD_3, and PGE_2 could stimulate proliferation of melanocytes. Yet, we have not fully understood a direct interaction between melanocytes and fibroblasts which should provide a basis for the characterization of the mechanism of normal and abnormal melanin pigmentation in the skin. Fibroblasts may directly affect the proliferation and differentiation of melanocytes.

Materials and Methods

Cell culture with growth medium; Melanocytes and fibroblasts were isolated from caucasian neonatal foreskin. Melanocytes were cultured in F-10 medium (F-10 nutrient mixture, Gibco BRL, USA) including phorbol 12-myristate 13-acetate (TPA), bFGF, 5% fetal bovine serum (FBS), and penicillin (100 U/m*l*)-streptomycin (100 μg/m*l*) (penicillin-streptomycin, Gibco, Grand Island, NY). Fibroblasts and SK-MEL23 were cultured in Dulbecco's modified Eagle medium (D-MEM, Gibco BRL, USA) with 10% FBS, and penicillin (100 U/m*l*)-streptomycin (100 μg/m*l*) (GIBCO, Grand Island, NY) in a humidified atmosphere containing 5% CO_2 at 37°C.

Fibroblast-conditioned medium (FCM); Human neonatal foreskin fibroblasts were cultured in D-MEM with 10% FBS for 3 days. They were washed with PBS (+), and cultured in D-MEM with heat-inactivated 2% FBS or F-10 medium with heat-inactivated 2% FBS for 2 days. After filtration, the medium was used for the supplement of fibroblast-conditioned medium. Control medium was D-MEM with heat-inactivated 2% FBS or F-10 medium with heat-inactivated 2% FBS.

Culture of melanocytes and melanoma cells on collagen gel; Collagen gels were prepared by mixing a collagen solution (KOKEN Cellgen, Japan), HEPES solution, RPMI 1640 medium, penicillin-streptomycin solution, 10% FBS, and 1N NaOH. One collagen gel included fibroblasts (final concentration: 2.5×10^5cell/m*l*), and the other one did not. Normal human epidermal melanocytes or SK-MEL23 cells were cultured on the collagen gel disks.

Cell proliferation assay; Melanocytes or SK-MEL23 cells were seeded on 96 wells culture plates with growth medium. At day 1 post-culture, the medium was changed to have various concentrations of FCM. The cell proliferation activities were measured through WST-1 assay.

WST-1 assay; This is a colorimetric assay for the quantification of cell proliferation and cell viability, based on the cleavage of the tetrazolium salt (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate; WST-1) by mitochondrial dehydrogenases in viable cells[5]. Cells were seeded on 96 well culture plates with growth medium. At day one post-seeding the dish was washed by PBS (+), and supplemented with 90 μl of the fibroblast-conditioned medium or control medium. Ten μl of WST-1 solution was added to the wells (final concentration; 10% W/W). After 2 or 3 hr culture, absorbance at 450 nm (reference: 690 nm) was measured by a spectrophotometric plate reader (Easy Reader, EAR 400 AT, SLT-Labinstruments, Austria).

Protein amount assay and DOPA-oxidase assay; Cells were cultured in growth medium for 1 day. The medium was washed by PBS(+), and changed by fibroblast-conditioned medium or control medium. At day 2 post-seeding, cells were washed by cold PBS(+) and detached by cell scraper. Cells were collected by centrifugation, and incubated in 0.1 M phosphate buffer (pH 6.8) including 1% Triton X and proteinase inhibitors. Cells were fragmented and mixed by a sonicator. Sample solutions were reacted with a protein dye (BioRad protein assay dye reagent concentrate, BIO-RAD) and the

absorbance at 595 nm was measured using a spectrophotometer (DU-65 spectrophotometer, Beckman, USA). Protein amounts were calculated from the result of the absorbance.

The sample solutions were mixed with a DOPA solution (1.65 μg/ml of L-3, 4-dihydroxyphenylalanine, Sigma, USA, final concentration 50% W[sample]/W[DOPA solution]) and incubated at 37°C. The absorbance at 475 nm was read at every 1 hr using a spectrophotometer (DU-65 spectrophotometer, Beckman, USA). The △O. D. 475 nm/min/mg protein was calculated from the results.

Attachment assay; Collagen-coated 96 well plates, non-coated plates, and plates with various concentrations of fibroblast-conditioned medium were prepared. Collagen solution (1.5 mg/ml) was coated on the plates for 1 hr at room temperature, and dried up. They were washed by PBS(−) twice, and further coated with 3% bovine serum albumin (BSA) for 1 hour at 37°C in order to prevent nonspecific attachment. They were washed by PBS(−) twice, again. Various concentrations of fibroblast-conditioned medium, which were diluted with control medium (D-MEM with 2% heat-inactivated FBS or F-10 with 2% heat-inactivated FBS), were coated on 96 well plates for 2 days at 37°C. They were further coated with 3% BSA for 1 hour at 37°C in order to prevent non-specific attachment. They were washed by PBS(−) twice.

Cells were seeded on the prepared plates, incubated for 1 or 2 hr at 37°C, washed with PBS(+), and incubated with growth medium including WST-1 solution (final concentration: 10% W/W) for 3 hours at 37°C. The absorbance was read at 450 nm (reference: 690 nm) using a spectrophotometer (DU-65 spectrophotometer, Beckman, USA).

Results

1. Morphological changes of human melanocytes and melanoma cells grown under different conditions

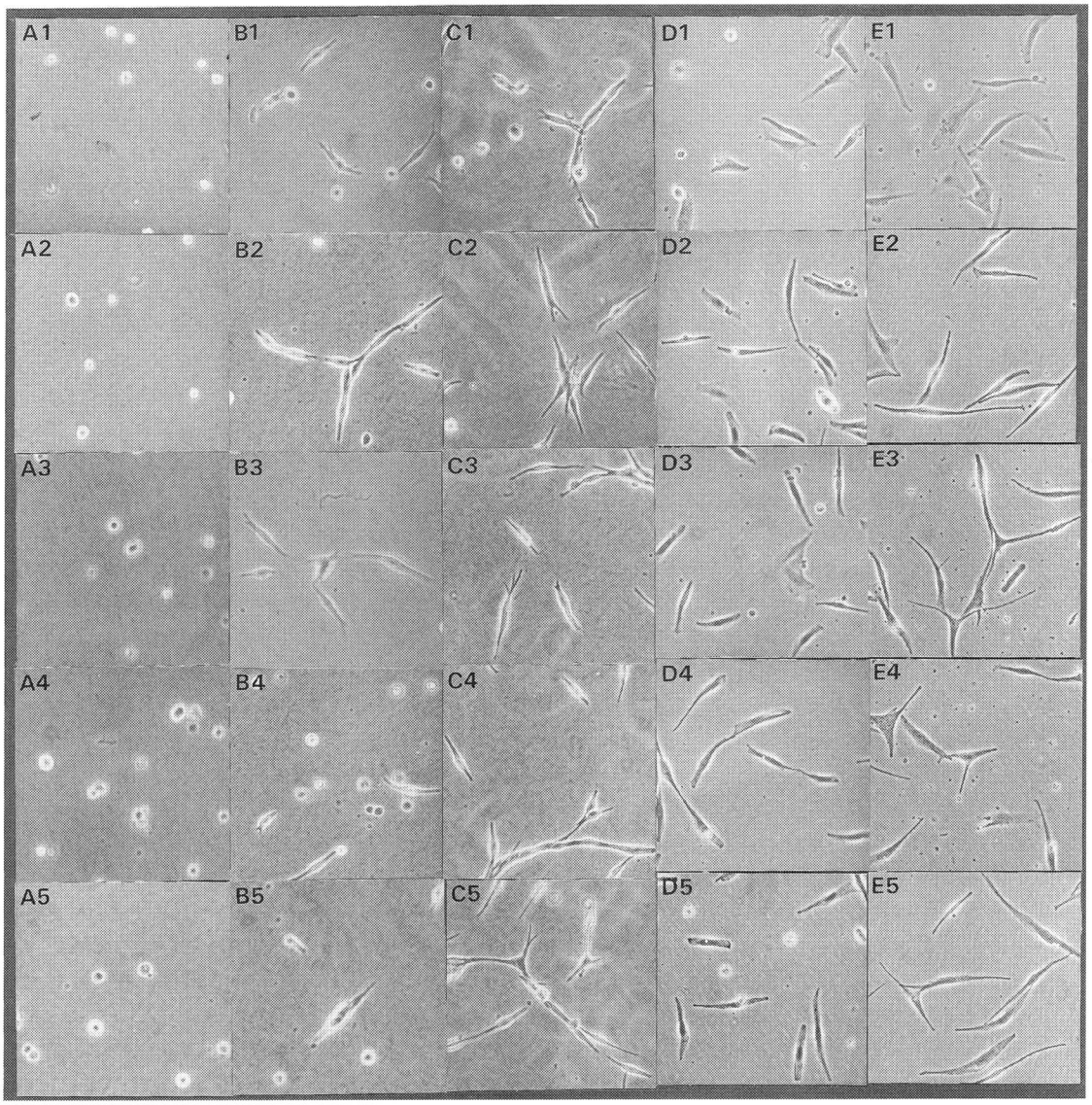
A. Normal human epidermal melanocytes (NHEM)

a. Collagen gel matrix without FCM; Normal human epidermal melanocytes, 5×10^5 cells per 3.5 cm petri dish were seeded on the collagen gel matrix. At 12 hr post-seeding, almost all of the melanocytes (approximately 100%) were still round and did not reveal any significant dendrites or perikaryon (Fig. 1-A1). Similarly there was no significant development of dendritic processes at 24 hr (Fig. 1-A2), 36 hr (Fig. 1-A3), 48 hr (Fig. 1-A4), and 72 hr (Fig. 1-A5) post-seeding.

b. Collagen gel matrix supplemented with 100% FCM; At 12 hr post-seeding, the melanocytes started to show dendricities, approximately 45-50% of the total NHEM (Fig. 1-B1). The dendrites were mostly slender and bipolar. Some dendritic processes (from approximately 25% of the total melanocytes), however, revealed split-tipped dendrites like small branches. The development of perikaryon was still poor. At 24 hr post-seeding, approximately 70% of melanocytes revealed dendritic processes, most of which were bipolar and slender, forming a fibroblast-like appearance (Fig. 1-B2). Remaining melanocytes, approximately 30%, were still round, without the obvious development of dendrites. At 36 hr post-seeding, remarkable differentiation of melanocytes was seen (Fig. 1-B3). Practically all of the melanocytes became dendritic and revealed either a fibroblast or an epithelioid cell-like shape, which was seen in approximately 80% and 20% of the total melanocytes, respectively. These melanocytes often revealed tripolar branching of dendritic processes. At 48 (Fig. 1-B4) and 72 hr (Fig. 1-B5) post-seeding, the melanocytes started rounding up, approximately 85 and 90% of the total melanocytes.

c. Collagen gel matrix and fibroblasts; Approximately 55% of melanocytes were dendritic at 12 hr post-seeding (Fig. 1-C1). Remaining melanocytes were still round, but some of them, approximately 15% of the total melanocytes, started to show buds and branching of dendrites. Some of the dendritic

melanocytes revealed well developed, enlarged perikaryon. Branching of dendritic processes was hardly seen in melanocytes grown with 100% FCM. At 24 hr post-seeding, approximately 90% of the total melanocytes became dendritic, showing fibroblast-like appearance (Fig. 1-C2). Some of the



Fig, 1 Morphological changes of normal human epidermal melanocytes grown under the different culture conditions.

A : seeded on the collagen gel matrix.

B : seeded on collagen gel matrix supplemented with 100% fibroblast-conditioned medium.

C : seeded on the collagen gel matrix including fibroblasts.

D : seeded on plastic petri dish.

E : seeded on plastic petri dish supplemented with 100% FCM.

The pictures were taken at 12 hr (Fig. A1, B1, C1, D1, and E1), 24 hr (Fig. A2, B2, C2, D2, and E2), 36 hr (Fig. A3, B3, C3, D3, and E3), 48 hr (Fig. A4, B4, C4, D4, and E4), and 72 hr (Fig. A5, B5, C5, D5, and E5) post-seeding.

melanocytes revealed the branching of dendritic tips with the development of triple dendrites. Similar findings were seen at 36 hr (Fig. 1-C3), 48 hr (Fig. 1-C4), and 72 hr (Fig. 1-C5) post-seeding.

d. Plain petri dish plate without FCM; Approximately 30% of the total melanocytes were round, without having any obvious dendrites or perikaryon at 12 hr post-seeding (Fig. 1-D1). Remaining melanocytes revealed either the flattening of whole cell body, but well developed perikaryon, having epithelioid cell-like appearance (approximately 35% of the total melanocytes) or bipolar perikaryon, having slender dendritic processes in the remaining 35% of melanocytes. Again the branching of the dendritic tip was hardly seen. At 24 hr post-seeding, the number of dendritic melancoytes increased, reaching approximately 60-70% of the total melanocytes (Fig. 1-D2). At 48 hr (Fig. 1-D4) and 72 hr (Fig. 1-D5) post-seeding, the number of dendritic cells did not appear to increase, but some melanocytes became epithelioid, approximately 40% of the total melanocytes.

e. Plain petri dish plate supplemented with 100% FCM; Only 5% of the total melanocytes were round at 12 hr post-seeding (Fig. 1-E1). The remaining 95% of melanocytes were either fibroblast- or epithelioid cell- like. Approximately 30% of these melanocytes showed bipolar, slender dendrites. The rest of the melanocytes, approximately 65%, revealed well-developed perikaryon. Some of these epithelioid melanocytes possessed branching of the perikaryon tip. At 24 hr post-seeding, the melanocytes started to show a bipolar, fibroblast-like appearance, reaching as much as 85-90% of the total melanocyte population (Fig. 1-E2). The same trend was seen at 36 hr (Fig. 1-E3), 48 hr (Fig. 1-E4), and 72 hr (Fig. 1-E5) post-seeding. These melanocytes, however, showed the most remarkable dendricity at 48 hr post-seeding.

The above mentioned results indicated that FCM has a significant effect in the morphological differentiation of NHEM. Therefore, the rest of the experiments were carried out only on the effect of FCM using various concentrations.

B. SK-MEL23 human pigmented melanoma cells

a. Plain petri dish plates supplemented with various concentrations of FCM; At 24 hours post-seeding, melanoma cells showed clustering of cells, being composed of round cells. This phenomenon of clustering and rounding-up of cells was most prominent at 0% FCM medium (Fig. 2-A1). With 10% FCM, they still showed cluster formation, but some of them, approximately 10% of the total of them had short dendrite tips (Fig. 2-A2). With 20% FCM, they showed the dendricity the same as that of 10% FCM culture (Fig. 2-A3). With increased concentrations of FCM at 40% (Fig. 2-A4), 60% (Fig. 2-A5), 80% (Fig. 2-A6), and 100% (Fig. 2-A7) FCM, melanoma cells gradually decreased the clustering of cells and increased the long dendrite formation. The number of dendritic cells increased, revealing longer processes. With 100% FCM, 90% of the total melanoma cells revealed dendricities, some of which were quite long.

b. Collagen gel matrix supplemented with various concentrations of FCM; In the culture medium, without any FCM, melanoma cells showed grouping and clustering of cells, which were composed of 60% round cells and 40% dendritic cells, but all of the cells possessed short dendrites (Fig. 2-B1). With 10% FCM, melanoma cells revealed basically the same findings as those with 0% FCM (Fig. 2-B2). With increased concentrations of FCM, 20% (Fig. 2-B3), 40% (Fig. 2-B4), 60% (Fig. 2-B5), 80% (Fig. 2-B6), and 100% (Fig. 2-B7), melanoma cells gradually possessed longer dendrites, and the number of dendritic cells increased. As a result, the ratio of dendritic melanoma cells in the total cell population increased 70%, 75%, 85%, 95%, and 100% with increased concentrations of FCM at 20%, 40%, 60%, 80%, and 100%, respectively.

2. Proliferation of human melanocytes and melanoma cells grown with and without FCM

A. Normal human epidermal melanocytes; The activity of cell proliferation was examined by util-

izing WST-1 colorimetric assay. Normal human epidermal melanocytes showed increased cell activity for the cell proliferation at day 1 post-FCM treatment (Fig. 3). Then thereafter there was a gradual decline in the activity from day 2 to day 5. An almost identical pattern of cell activity was seen in normal human epidermal melanocytes grown in the medium without FCM. In order to further investigate if there is any effect of FCM on cell proliferation, concentration of FCM was changed from 10% to

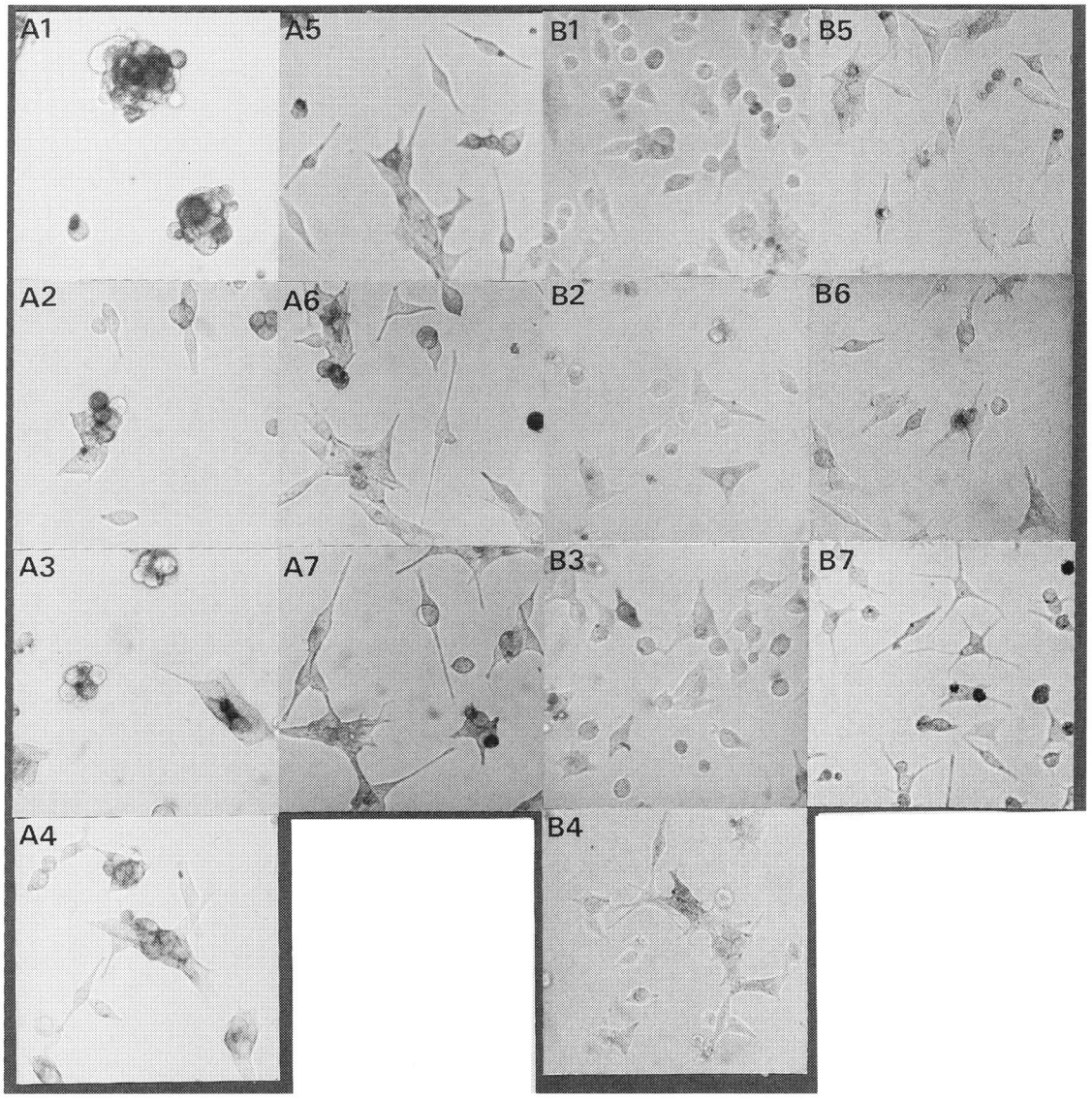


Fig. 2 Morphological changes of SK-MEL23 cells grown under the different culture conditions.
A : seeded on the plain petri dish supplemented with various concentrations of FCM, i. e., 0% (Fig. A1), 10% (Fig. A2), 20% (Fig. A3), 40% (Fig. A4), 60% (Fig. A5), 80% (Fig. A6), and 100% (Fig. A7).
B : seeded on the collagen-coated petri dish supplemented with various concentrations of FCM, i. e., 0% (Fig. B1), 10% (Fig. B2), 20% (Fig. B3), 40% (Fig. B4), 60% (Fig. B5), 80% (Fig. B6), and 100% (Fig. B7).
The pictures were taken at 24 hr post-seeding.

100% (Table 1). Again there was no significant difference in the cell proliferation activity, indicating that there is not any direct effect of FCM on cell proliferation.

B. SK-MEL23 human melanoma cells; Human melanoma cells of SK-MEL23 also did not reveal any significant difference between two cell groups grown in the medium with and without FCM (Fig. 4). Human melanoma cells, however, revealed the maximum peak of cell proliferation activity at day 3 post-treatment. Similar to normal human epidermal melanocytes, human melanoma cells were treated

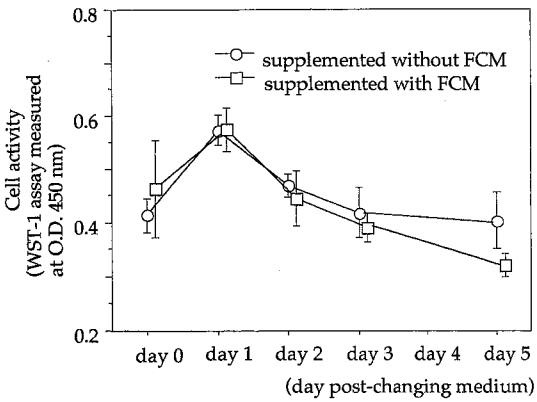


Fig. 3 Cell proliferation assay of NHEM supplemented with or without FCM. No significant difference is present.

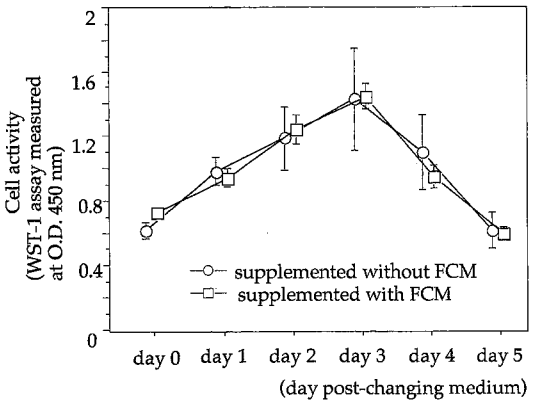


Fig. 4 Cell proliferation assay of SK-MEL23 melanoma cells supplemented with or without FCM. No significant difference is present.

Table 1 *Cell proliferation assay of NHEM supplimented with various concentrations of FCM*

FCM concentration	cell activity[a)]	S. D.[b)]	p-value[c)]
0	0.160	0.028	—
10%	0.165	0.032	0.6644
20%	0.168	0.035	0.5739
40%	0.180	0.030	0.1305
60%	0.188	0.039	0.0759
80%	0.175	0.019	0.1465
100%	0.181	0.022	0.0574

a ; measured at O. D. 450 nm, b ; standard deviations, c ; by unpaired t-test

Table 2 *Cell proliferation assay of SK-MEL23 supplimented with various concentrations of FCM*

FCM concentration	cell activity[a)]	S. D.[b)]	p-value[c)]
0	1.012	0.090	—
10%	1.031	0.055	0.5572
20%	1.067	0.080	0.1634
40%	1.052	0.074	0.2892
60%	1.050	0.053	0.2591
80%	0.944	0.091	0.1117
100%	0.950	0.051	0.0767

a ; measured at O. D. 450 nm, b ; standard deviations, c ; by unpaired t-test

with the medium containing a different concentration of FCM, ranging from 10% to 100%, and there was no alteration in the cell proliferation activity (Table 2).

3. Attachment of human melanocytes and melanoma cells grown in different culture conditions

A. Normal human epidermal melanocytes (NHEM)

a. Plain plates coated with various FCM concentrations; The attachment activity of the cell was examined by utilizing WST-1 colorimetric assay. The results were expressed as a relative cell attachment (cell population) against the total attachment (cell population) of seeded cells. Normal human epidermal melanocytes showed increased cell attachment to 96 well culture plates coated with various concentrations of FCM (Table 3). On the plate with 0% FCM coating, only 54% of the total melanocytes attached to the plate for the first 2 hr post-seeding. On the plate with 10% FCM coating, attached melanocytes increased, reaching the ratio of 70% of the total melanocytes. On the plate with 20 to 100% FCM coatings, approximately 80 to 95% of the total melanocytes attached. The attached cell number showed a tendency towards a dose-dependent increase.

b. Plain plates supplemented with medium of various FCM concentrations; Normal human epidermal melanocytes showed increased cell attachment to 96 well culture plates supplemented with various FCM concentrations (Table 4). In the medium with 0% FCM, only 55% of the total melanocytes attached to the plate for the first 2 hr post-seeding. In 10 to 100% FCM media, approximately 95 to 100% of the total melanocytes attached to the plates.

c. Collagen-coated plates supplemented with medium of various FCM concentrations; Normal human epidermal melanocytes showed increased cell attachment to 96 well collagen-coated culture plates

Table 3 *Attachment assay of NHEM on plates coated with various concentrations of FCM*

FCM concentration	ratio of attached cell	S. D.[a)]	p-value[b)]
0	0.536	0.024	—
10%	0.682	0.079	0.0015
20%	0.827	0.057	<0.0001
40%	0.900	0.101	<0.0001
60%	0.914	0.127	<0.0001
80%	0.847	0.068	<0.0001
100%	0.941	0.066	<0.0001

a ; standard deviations, b ; by unpaired t-test

Table 4 *Attachment assay of NHEM on plates supplemented with various concentrations of FCM*

FCM concentration	ratio of attached cell	S. D.[a)]	p-value[b)]
0	0.549	0.031	—
10%	0.949	0.066	<0.0001
20%	0.976	0.086	<0.0001
40%	0.996	0.097	<0.0001
60%	0.970	0.111	<0.0001
80%	1.006	0.108	<0.0001
100%	0.997	0.100	<0.0001

a ; standard deviations, b ; by unpaired t-test

supplemented with various concentrations of FCM (Table 5). In the 0% FCM media, 88% of the total melanocytes attached to the plates for the first 2 hr post-seeding. In the 10 to 100% FCM media, approximately 96 to 100% of the total melanocytes attached to the collagen-coated plates.

B. SK-MEL23 human melanoma cells

a. Plain plates coated with various FCM concentrations; Human melanoma cells of SK-MEL23 showed increased cell attachment to 96 well culture plates coated with various concentrations of FCM (Table 6). On the plates coated with 0% FCM, 84% of the total melanoma cells were attached in the first 2 hr post-seeding. On plates coated with 10 to 60% FCM, the number of attached melanoma cells did not increase, i. e., approximately 83 to 89%. On plates, coated with 80 and 100% FCM, approximately 95 to 100% of the total melanoma cells revealed the cell attachment.

b. Plain plates supplemented with medium of various FCM concentrations; Human melanoma cells showed increased cell attachment to 96 well culture plates supplemented with various concentrations of FCM (Table 7). With 0% FCM, only 51% of the total melanoma cells attached on plates for the first 2 hr post-seeding. With 10 to 60% FCM, only 52 to 55% of the total melanoma cells attached on plates. With 80 to 100% FCM, approximately only 66 to 68% of the total melanoma cells attached on plates. On the whole, the ratio of the attached melanoma cell was much lower than that of normal human epidermal melanocytes.

c. Collagen-coated plates supplemented with medium of various FCM concentrations; Human melanoma cells showed increased cell attachment to 96 well collagen-coated culture plate supplemented with various concentrations of FCM (Table 8). With 0% FCM media, 82% of the total melanoma cells attached on plates for the first 2 hr post-seeding. With 10% FCM media, 88% of the total melanoma

Table 5 *Attachment assay of NHEM on collagen-coated plates supplemented with various concentrations of FCM*

FCM concentration	ratio of attached cell	S. D.[a)]	p-value[b)]
0	0.879	0.068	—
10%	0.962	0.030	0.0678
20%	0.987	0.028	0.0264
40%	0.977	0.025	0.0353
60%	0.983	0.047	0.0462
80%	0.998	0.029	0.0188
100%	1.006	0.025	0.0130

a ; standard deviations, b ; by unpaired t-test

Table 6 *Attachment assay of SK-MEL23 on plate coated with various concentrations of FCM*

FCM concentration	ratio of attached cell	S. D.[a)]	p-value[b)]
0	0.841	0.019	—
10%	0.827	0.032	0.3834
20%	0.849	0.026	0.5930
40%	0.872	0.031	0.0670
80%	0.946	0.111	0.0464
100%	1.007	0.047	<0.0001

a ; standard deviations, b ; by unpaired t-test

Table 7 *Attachment assay of SK-MEL23 on plates supplemented with various concentrations of FCM*

FCM concentration	ratio of attached cell	S. D.[a)]	p-value[b)]
0	0.509	0.057	—
10%	0.525	0.033	0.5732
20%	0.549	0.044	0.2055
40%	0.519	0.062	0.7699
60%	0.520	0.035	0.7045
80%	0.656	0.046	0.0006
100%	0.676	0.061	0.0006

a ; standard deviations, b ; by unpaired t-test

Table 8 *Attachment assay of SK-MEL23 on collagen-coated plates supplemented with various concentrations of FCM*

FCM concentration	ratio of attached cell	S. D.[a)]	p-value[b)]
0	0.822	0.050	—
10%	0.877	1.220	0.3308
20%	0.936	0.051	0.0049
40%	0.901	0.049	0.0206
60%	0.966	0.061	0.0012
80%	0.986	0.055	0.0003
100%	0.978	0.050	0.0003

a ; standard deviations, b ; by unpaired t-test

cells attached. With 20 to 100% FCM media, approximately 90 to 99% of the total melanoma cells attached on collagen-coated plates. The ratio of attached cell population had a tendency of a dose-dependent increase.

4. Tyrosinase activity of human melanocytes and melanoma cells grown in the medium with various FCM concentrations

A. Normal human epidermal melanocytes ; The tyrosinase activities were presented as △O. D. 475 nm/min/mg total protein. Normal human epidermal melanocytes showed a decreased tyrosinase activity with a dose-dependent tendency. Melanocytes cultured in the media of 0% FCM concentration had 7.02×10^{-3} △O. D. 475 nm/min/mg total protein. Tyrosinase activities were decreased to 5.78×10^{-3}, 5.9×10^{-3}, 3.85×10^{-3}, and 2.15×10^{-3}, in the medium 10%, 20%, 50%, and 100% FCM concentrations, respectively (Table 9).

B. SK-MEL23 human melanoma cells ; Tyrosinase activities ranged from 15.7×10^{-3} to 19.5×10^{-3}, in the medium with 0% to 100% FCM concentrations. They did not have any significant differences, as compared to the result of normal melanocytes (Table 10).

Discussion

In this study, we are addressing the role of skin fibroblasts in the growth and differentiation of melanocytes using human neonatal foreskin melanocytes and fibroblasts as a model. We measured the dendrite formation, plate/dish attachment, proliferation rate, and tyrosinase activity of normal human epidermal melanocytes (NHEM) using human pigmented melanoma cells (SK-MEL23) as controls.

Table 9 *Tyrosinase activity (DOPA-Oxidase) assay of NHEM supplimented with various concentrations of FCM*

FCM concentration	tyrosinase activity	S. D.[b)]	p-value[c)]
0	7.02	4.5×10^{-4}	—
10%	5.78	2.0×10^{-4}	0.012
20%	5.90	5.3×10^{-4}	0.0495
50%	3.85	6.7×10^{-4}	0.0003
100%	2.15	3.3×10^{-4}	0.0001

a ; measured at O. D. 475 nm$\times10^{-3}$/min/mg protein, b ; standard deviations, c ; by unpaired t-test

Table 10 *Tyrosinase activity (DOPA-Oxidase) assay of SK-MEL23 supplimented with various concentrations of FCM*

FCM concentration	tyrosinase activity	S. D.[b)]	p-value[c)]
0	1.89	1.13×10^{-3}	—
10%	1.93	9.30×10^{-4}	0.6606
20%	1.57	5.20×10^{-4}	0.0109
40%	1.79	5.30×10^{-4}	0.2311
60%	1.95	1.03×10^{-3}	0.5362
80%	1.91	6.00×10^{-4}	0.8437
100%	1.77	8.20×10^{-4}	0.1918

a ; measured at O. D. 475 nm$\times10^{-2}$/min/mg protein, b ; standard deviations, c ; by unpaired t-test

These cells were cultured as monolayer on normal plastic petri dishes with and without collagen coating in the presence of fibroblasts or fibroblast-conditioned medium (FCM). Our results indicate that the conditions with fibroblasts or FCM stimulate the morphological differentiation of NHEM and SK-MEL23 melanoma cells to develop longer dendrites, their perikaryon also becoming much larger in comparison with those controls grown on the plastics. This morphological change can result from cytokines and/or extracellular matrix (ECM) produced by fibroblasts. Importantly, the degrees of morphological alterations of NHEM and SK-MEL23 are different. The condition with FCM alone introduced dramatic changes in the dendrite formation and plate/dish attachment of NHEM, however, it caused much less alteration on SK-MEL23 cells. The FCM induced elongation of dendrites and their branching formation. It appeared that this difference derived from the intrinsic characteristics (property) of these cell lines. SK-MEL23 cells derive from a pigmented malignant melanoma cell line, and they basically show a high metastatic activity and, therefore, a low attachment activity to their environment. Interestingly, the morphological alteration by FCM decreased at day 3 post-FCM addition (the data is not shown). The effectors in FCM, produced by fibroblasts, appear to lose their activities within 3 day-culture. In general, many cytokines or extracellular matrix (ECM) proteins can be degraded by enzymes (e. g., protease) within several days. Cell proliferation assays exhibited no effect of FCM on the NHEM and SK-MEL23 cells. FCM can not make any changes on the cell growth activity of NHEM and SK-MEL23 cells compared with non-FCM control medium. NHEM showed cell growth peak at day 1 after medium change, on the other hand, SK-MEL23 cells showed this peak at day 3 after medium change. This appearance may indicate that SK-MEL23 cells can be resistant to the culture medium condition and grow independently[6,7,8,9], but that NHEM needs to have some environmental

growth-promoting agents. In this study, we used slow-growth medium. Therefore, SK-MEL23 cells could proliferate for several days, but NHEM could not. FCM-coated petri dish enhanced the attachment of NHEM and SK-MEL23 cells in the dose-depended manner. When FCM was supplemented in attachment cultuer plate, FCM increased the attachment of NHEM and SK-MEL23 cells on the collgen-coated or ordinary petri dish. ECM produced dy fibroblasts induced cell attachment to the dish. Melanoma cells have autocrine activity and proliferate by themselves. The culture condition with FCM decreased tyrosinase (DOPA-oxidase) activity of NHEM, however, there was no significant change SK-MEL23 cells. Northern blotting could not show a significant difference in the tyrosinase mRNA production among the various FCM concentrations (the data is not shown). At the present, we do not have a clear explanation of the reverse response of functional activity (tyrosinase activity) to morphological differentiation (dendrite formation) by melanocytes. In our observations, the presence of fibroblasts or FCM did not induce the proliferation of normal human epidermal melanocytes. The ^{3}H thymidine uptake study also shows that melanocyte proliferation is not changed (the data is not shown). On the other hand, human melanoma cells showed fewer morphological changes after exposure to FCM, but an increased cell number, indicating that melanoma cells can proliferate without extrinsic promoters, while NHEM requires them.

A number of previous studies showed the effect of fibroblasts to melanocyte growth and differentiation[10,11]. Fibroblasts are indicated to produce a variety of cytokines and extracellular matrix, including interleukin-1 (IL-1), interleukin-6 (IL-6)[12], interleukin-8 (IL-8)[13], basic fibroblast growth factor (bFGF)[14], transforming growth factor-α (TGF-α), tumor necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), granulocyte/macrophage colony stimulating factor (GM-CSF), hepatocyte growth factor/scatter factor (HGF/SF)[15], nerve growth factor (NGF)[16,17], interferon-α (IFN-α), and interferon-β (IFN-β). Fibroblasts also secrete type-I collagen, type-III collagen, fibronectin, and laminin. These factors should have a direct or indirect effect on melanocyte morphology, proliferation, and differentiation.

The alteration of melanocyte dendricities is known to be provided by a number of growth factors. Prostaglandin E_2 (PGE_2)[18], NGF[19], endothelin-1 (ET1)[20], TPA, cAMP, K-CM[21], UVR, and VD_3 are reported to elongate the cytoplasmic processes and dendrites of melanocyte. αMSH, diacylglycerol (DAG), IFN-α[22], IFN-β[22] and bFGF do not appear to have any effect on these dendricities. In contrast, leukotrien C_4 (LTC_4), IFN-γ[22], and TNF-α are reported to decrease dendricities. Furthermore, LTC_4[23], ET-1[24], bFGF[25,26], LTD_4[23], cAMP[27] αMSH, K-CM[21], IL-8, and HGF/SF[28] are known to stimulate melanocyte proliferation. αMSH[27], DAG, NGF, IFN-α[22], IFN-γ[22], and PGE_2[18] can not stimulate melanocyte proliferation. However, IL-1[29], IL-6[29], TNF-α[29], UVR, TGF-β[30], and IFN-$\alpha\beta$[22]γ decrease melanocyte proliferation. LTB_4[31], PGD_2, PGE_2, ET-1[24], TPA, cAMP, K-CM[21], DAG, VD_3, UVR, and αMSH[32] have a positive effect on melanogenesis of melanocytes, while IL-1[29], IL-6[29], TNF-α[29], and LTC_4[31] has a negative effect (αMSH, NGF, and bFGF have no effect on melanogenesis). As described above, cytokines have complicated function to melanocytes. From our study we are unable to address which one of the cytokines could cause the observed alterations of melanocytes. The ECM has been shown to provide signals for cell differentiation and proliferation[33]. In the *in vitro* condition, ECM strongly modifies cell morphology and attachment efficacy. Gilchrest *et al.*[34] found that crude ECM components (which included fibronectin, collagens type I/III or IV, or laminin), as well as bovine serum albumin, enhanced the attachment and spreading of human epidermal melanocytes, but purified ECM proteins failed to stimulate the ECM effect. Ranson *et al.*[35] reported that melanocytes cultured on ECM coated plates developed flatter and larger cell bodies and produced more melanin pigments compared to those of melanocytes cultured on uncoated plates. Buffey[36] showed

that FCM enhanced the proliferation and melanogenesis of melanocytes. They also indicated that the biological effect of FCM was dependent on the activity of growth-promoting factors which FCM contained. ECM proteins can affect cell proliferation and differention through their binding to the cell surface receptors of integrin families. They can also act as a reservoir for smaller mitogens produced by cell in the matrix[37]. Our future investigations should focus on the subject as to which one of the cytolines or ECMs in FCM has provided the major morphological and functional alterations of melanocytes observed in this study.

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正常ヒト表皮メラノサイト（NHEM）とヒト黒色腫細胞株SK-MEL23の細胞増殖と分化に対する線維芽細胞培養液上清（FCM）の影響

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我々の目的は，再構成表皮モデルであるepidermal melanin unit (EMU) を用いて，表皮メラノサイトの増殖・分化に対する真皮線維芽細胞の役割を研究することである．この目的を達成するために，我々は正常ヒト表皮メラノサイト（NHEM）とヒト黒色腫細胞株SK-MEL23の細胞増殖と分化に対する線維芽細胞培養液上清（FCM）の影響を調べた．

タイプⅠコラーゲン上では、NHEMとSK-MEL23細胞は短い突起を伸ばすのみであった．一般にFCMには線維芽細胞より分泌される細胞外基質・サイトカインが含まれていると考えられている．FCMはNHEMの突起形成能とコラーゲンへの接着能を著名に増強させたが，SK-MEL23細胞に対しては軽度の増強しか示さなかった．一方，FCMはNHEMとSK-MEL23細胞に対し増殖促進作用を示さなかった．また，FCM処理によって色素産生細胞の基本的な機能酵素であるtyrosinaseは，NHEMでは活性が抑制されたがSK-MEL23では抑制されなかった．

これらの所見により，線維芽細胞から分泌される細胞外基質・サイトカインが（a）正常ヒト表皮メラノサイト（NHEM）の機能的・形態的分化に対し重要な役割を持っていること及び（b）SK-MEL23細胞にはあまり影響しないこと，これら2点が分かった．