Ferrous Ferric Chloride Stimulates the Proliferation and Differentiation of Cultured Keratinocytes and Melanocytes in the Epidermis of Neonatal Mouse Skin

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Ferrous ferric chloride (FFC) is a special aqueous iron which is a complex of ferrous chloride and ferric chloride and has a function in both oxidation and reduction. FFC is thought to stimulate the cellular function of living organisms. Although FFC is reported to stimulate the function of red blood cells, it has not been determined whether FFC stimulates the function of skin cells. To understand the role of FFC in the function of skin cells, we studied its effects on the proliferation and differentiation of keratinocytes and melanoblasts or melanocytes. FFC was added to a serum-free culture of neonatal mouse epidermal cells and its effects on the proliferation and differentiation of keratinocytes and melanoblasts or melanocytes were investigated. FFC stimulated the proliferation and differentiation of keratinocytes and melanoblasts or melanocytes. The proliferation of keratinocytes and that of melanoblasts or melanocytes was stimulated to the same extent (a two-fold increase), suggesting that the proliferation of the two types of cells constituting the epidermis may be equally stimulated by FFC. These results suggest that FFC may activate skin function by promoting cell renewal via the stimulation of the proliferation and differentiation of keratinocytes and melanoblasts or melanocytes.

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INTRODUCTION

Ferrous ferric chloride (FFC) is a special aqueous iron which is a complex of ferrous chloride and ferric chloride,[1] and has the function of both oxidation and reduction. FFC is thought to stimulate the cellular function of living organisms by changing water in cells from the oxidative to antioxidative state. Water containing FFC can be obtained by immersing FFC ceramic beads manufactured by Akatsuka Cooperation (Mie, Japan) in water. These FFC ceramic beads are prepared by heating hardened soil with FFC solution to *ca.* 1200°C. FFC ceramic beads are made of porous ceramic treated with FFC solution. FFC ceramic-treated water (FFC water) contains various minerals such as Ca, Mg, K, Na, Fe, Cu, *etc.*, in addition to FFC.

Brain reported that *ca.* 10 mg of Fe in mass was released from 1 g of FFC ceramic beads after incubating the ceramic in H_2O for 1 hr using neutron activation analysis.[2] He also reported that hemoglobin contents and red blood cell numbers in rats were increased by giving FFC water to them everyday.[2] His report suggests that FFC may be released from FFC ceramic and FFC acts on red blood cells to stimulate their biological activities. However, it is unknown at present whether FFC acts on skin cells to stimulate their biological function. To understand the role of FFC in the function of skin cells, we studied its effects on the proliferation and differentiation of keratinocytes and melanoblasts or melanocytes. A serum-free culture system of primary keratinocytes[3] and melanoblasts or melanocytes[4,5] derived from newborn mouse skin of the C57BL/10JHir strain has been developed in our laboratory. In this study, FFC was added to the serum-free culture medium and tested for its ability to stimulate the proliferation and differentiation of keratinocytes and melanoblasts or melanocytes.

MATERIALS AND METHODS

Mice —— Strain C57BL/10JHir mice (house mouse) were given water and a commercial diet, OA-2 (Clea Japan, Tokyo, Japan), *ad libitum*.

They were maintained at 24 ± 1°C with 40–60% relative humidity, and 12 hr of fluorescent light was provided daily. The present study was approved by the Ethics Committee of the National Institute of Radiological Sciences in accordance with the guidelines of the National Institutes of Health.

Primary Culture —— Unless stated otherwise, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The method for obtaining epidermal cell suspensions from dorsal skins of 0.5-day-old mice was reported previously.[3] Disaggregated epidermal cell suspensions were pelleted by centrifugation and suspended in Ham's F-10 medium (Sigma). After centrifugation, cell pellets were resuspended in one of four culture media.[6] Melanoblast-defined medium (MDM) consisted of Ham's F-10 plus insulin (bovine) 10 μg/ml, bovine serum albumin 0.5 mg/ml (fraction V), ethanolamine 1 μM, phosphoethanolamine 1 μM, sodium selenite 10 nM, penicillin G 100 U/ml, streptomycin sulfate 100 μg/ml, gentamycin sulfate 50 μg/ml, and amphotericin B 0.25 μg/ml. Melanocyte-differentiation medium (MDMM) consisted of MDM supplemented with α-melanocyte-stimulating hormone (MSH) 100 nM. Melanocyte-proliferation medium (MDMD) consisted of MDM supplemented with dibutyryl adenosine 3′ : 5′-cyclic monophosphate (DBcAMP) 0.5 mM. Melanoblast-proliferation medium (MDMDF) consisted of MDM supplemented with DBcAMP 0.5 mM plus basic fibroblast growth factor (bFGF) 2.5 ng/ml. The same lots of these supplements were used in this study. The cells in the epidermal cell suspension were counted in a hemocytometer chamber and plated onto dishes coated with type I collagen (Becton Dickinson, Bedford, MA, U.S.A.) at an initial density of 1×10^6 cells/35–mm dish (1.04×10^5 cells/cm^2). Cultures were incubated at 37°C in a humidified atmosphere composed of 5% CO_2 and 95% air (pH 7.2). The medium was replaced with a fresh one four times a week. After 14 days, almost pure cultures of melanoblasts or melanocytes were obtained.

FFC can be made as follows.[1] Ferric chloride ($FeCl_3 \cdot 6\,H_2O$) is dissolved in a mixed solution of ammonium formate 2 M, hydroxylamine 1 M, and formamide 1 M to a final concentration of 1 M and then diluted with H_2O to 10^{-8}–10^{-14} mM. Another $FeCl_3 \cdot 6\,H_2O$ (1 g/10 ml solution) is dissolved in the solution and then gradually heated at 100°C until FFC can be crystallized.[1] The proportion of Fe(II) to Fe(III) was 4 : 6 (10^{-8} mM solution), 6 : 4 (10^{-12} mM solution), and 7 : 3 (10^{-14} mM solution). The analysis using ion-exchange chromatography and X-ray diffractometer suggests that Fe(II) and Fe(III) form dimers.[1] In the case of FFC, 1 day before the initiation of culture, 60 g of FFC ceramic beads manufactured by Akatsuka Cooperation was placed in 2 l of deionized and distilled water (DDW) for 12 hr in a styrene foam box at room temperature, and this DDW is referred to as FFC-DDW. In FFC-DDW, FFC in addition to various minerals such as Ca, Mg, K, Na, Fe, Cu, *etc*., were present.[2] Further, 1 day before the initiation of culture, 60 g of pawa stone ceramic (PSC) beads manufactured by Watanabe Corporation (Tokyo, Japan) was similarly placed in DDW, and this DDW is referred to as PSC-DDW. In PSC-DDW, similar minerals such as Ca, Mg, K, Na, Fe, Cu, *etc*., were present, but not FFC. Powder medium of F-10 (Sigma) was dissolved in DDW, FFC-DDW, and PSC-DDW, MDM, MDMM, MDMD, and MDMDF were prepared from F-10, and the effects of FFC or PSC on the proliferation and differentiation of keratinocytes and melanoblasts or melanocytes were examined. In some cases, cultured primary melanoblasts or melanocytes in MDM and MDMDF or MDMM and MDMD were cultured for another 7 days in MDM and MDMDF or MDMM and MDMD prepared with DDW or FFC-DDW from 14 days (keratinocyte depletion).

Assays of Proliferation and Differentiation —— The number of keratinocytes per colony was counted after 2 days of primary culture from 10 randomly chosen microscopic fields under phase-contrast microscopy. About 100 keratinocyte colonies were surveyed in each experimental group. Keratinocyte colonies consisting of more than three keratinocytes were targeted for counting and were scored. The size of single keratinocytes or of keratinocytes from keratinocyte colonies was used to assess keratinocyte differentiation. The number of melanoblasts and melanocytes per dish was determined by phase-contrast and bright-field microscopy, and the calculation was based on the average number of cells from 10 randomly chosen microscopic fields covering an area of 0.581 mm^2. The method for determining the mitotic indices was as follows. The total number of melanoblasts and melanocytes was measured similarly for each dish, and the total number of melanoblasts and melanocytes in mitotic division was counted per dish under phase-contrast and bright-field microscopy. Mitotic indices were estimated by cal-

culating the proportion of mitotic melanoblasts and melanocytes to the total number of melanoblasts and melanocytes (%). The statistical significance of differences in the number of melanoblasts and melanocytes or in the percentage of melanocytes in the melanoblast-melanocyte population was determined using Student's *t*-test for comparing groups of equal size.

Identification of Melanocytes and Melanoblasts —— Bipolar, tripolar, dendritic, polygonal, or epithelioid cells, as seen under phase-contrast microscopy, which contained brown or black pigment granules, as observed under bright-field microscopy, were scored as differentiated melanocytes (pigmented melanocytes). The number of differentiated melanocytes was comparable to the number of L-3,4-dihydroxyphenylalanine (dopa)-positive melanocytes.[7] In contrast, bipolar, tripolar, dendritic, or polygonal cells, as seen under phase-contrast microscopy, which contained no pigment and were negative for dopa staining, as observed under bright-field microscopy, were scored as melanoblasts. These cells were stained with the combined dopa-premelanin reaction (combined dopa-ammoniacal silver nitrate staining).[8,9] This staining reveals preferentially undifferentiated melanoblasts that contain stage I and II melanosomes in addition to tyrosinase (TYR)-containing differentiated melanocytes. The ammoniacal silver nitrate reaction specifically reveals unmelanized melanosomes as well as melanized melanosomes in melanocytes, and the metallic silver particles are deposited with a high degree of selectivity.[10,11] Melanoblasts were also stained with antibodies to TYR-related protein (TRP)-1 and TRP-2 (or dopachrome tautomerase, DCT).[12] A "melanoblast" was defined as an unpigmented cell that has no TYR activity. Melanoblasts cultured in MDMM, MDMD, or MDMDF were stained using the combined dopa-premelanin reaction as well as using antibodies for TRP-1 and TRP-2.[12] However, melanoblasts cultured in MDM were negative for the combined dopa-premelanin reaction as well as to TRP-1 and TRP-2.[12]

RESULTS AND DISCUSSION

Effects of FFC on the Proliferation and Differentiation of Keratinocytes and Melanoblasts or Melanocytes

MDM is used for defining and maintaining melanoblasts. Since MDM does not contain melanogens, it can be determined using this medium whether FFC exhibits differentiation-stimulating activity toward melanoblasts in the primary culture of epidermal cell suspensions. When the epidermal cell suspensions were cultured in MDM, undifferentiated bipolar, tripolar, or dendritic melanoblasts were in contact with adjacent keratinocyte colonies (Fig. 1A) through a dendrite process within 1–2 days. After 3 days, the melanoblasts gradually increased in number. After 7–8 days, the keratinocyte colonies were gradually decreased, and by 14 days, almost all keratinocytes had died and pure cultures of melanoblasts (Fig. 1C) were obtained. In the case of MDM with FFC, large keratinocyte colonies with numerous enlarged keratinocytes (Fig. 1B) were observed. Although the number of melanoblasts failed to increase (Table 1A), the percentage of differentiated melanocytes in the melanoblast-melanocyte population (Fig. 1D and Table 1A) increased (> 40%). These results suggest that FFC can stimulate the differentiation of melanocytes in the medium that defines and maintains melanoblasts in normal circumstances.

MDMM containing α-MSH is used for stimulating melanocyte differentiation. Since α-MSH is known to stimulate melanocyte differentiation *in vivo* and *in vitro*,[6] it can be determined using this medium whether FFC exhibits differentiation-stimulating activity toward melanoblasts in cooperation with α-MSH. When the epidermal cell suspensions were cultured in MDMM, pigment-producing differentiated melanocytes appeared around keratinocyte colonies within 2–3 days and increased in number. After 14 days, almost all the keratinocytes had died and cultures of differentiated melanocytes were obtained (Fig. 2A). In the case of MDMM with FFC, large keratinocyte colonies with numerous enlarged keratinocytes were observed similar to those cultured in MDM with FFC. Although the number of melanoblasts and melanocytes failed to increase (Table 1B), melanocyte differentiation was stimulated (Table 1B) and, in addition, dendritogenesis, cell expansion, and pigmentation (Fig. 2B) were increased. These results suggest that FFC can stimulate the differentiation, dendritogenesis, and pigmentation of melanocytes in cooperation with α-MSH.

MDMD containing DBcAMP is used for stimulating both the proliferation and differentiation of melanocytes. Since DBcAMP is known to stimulate both proliferation and differentia-

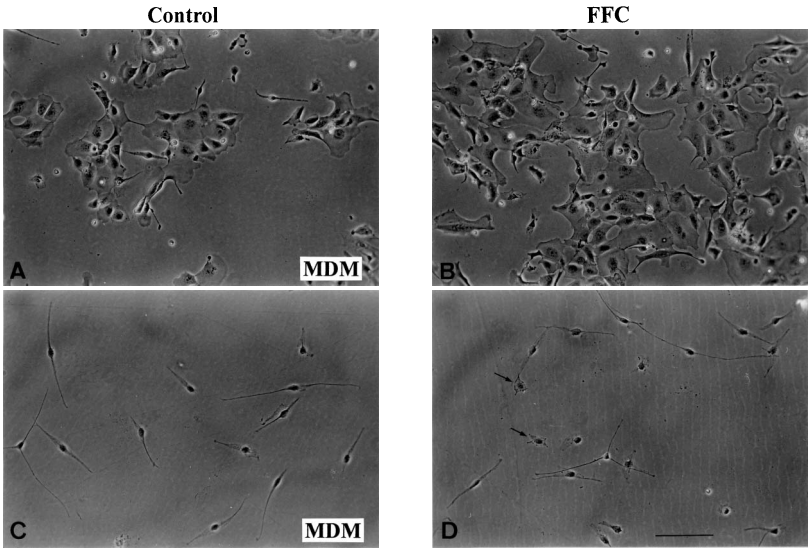

Fig. 1. Effects of FFC on the Proliferation and Differentiation of Mouse Epidermal Keratinocytes and Melanoblasts or Melanocytes in Serum-Free Primary Culture (MDM)

Epidermal cell suspensions derived from mouse skin were cultured in MDM (A, C) or in MDM with FFC (B, D). After 2 days, colonies of keratinocytes were observed in control cultures (A). In MDM with FFC, large keratinocyte colonies with numerous enlarged keratinocytes were observed (B). After 7 days, keratinocytes decreased in number and after 14 days, pure cultures of melanoblasts were obtained in control cultures (C). However, in the cultures with FFC, many differentiated melanocytes (D, arrows) were observed. Phase-contrast microscopy. Bar, 100 μm.

Table 1. Effects of FFC and PSC on the Proliferation and Differentiation of Mouse Epidermal Melanocytes

Medium	No. of melanoblasts and melanocytes/35–mm dish × 10^4			Percentage of melanocytes in the melanoblast-melanocyte population		
	1 day	7 days	14 days	1 day	7 days	14 days
(A) MDM	0.36 ± 0.04	3.52 ± 0.82	3.74 ± 0.72	1.28 ± 1.28	4.08 ± 1.60	4.60 ± 0.61
MDM + FFC	0.40 ± 0.05	3.69 ± 0.62	3.94 ± 0.47	2.56 ± 1.31	27.19 ± 6.44*	43.69 ± 1.65*
(B) MDMM	0.62 ± 0.23	4.23 ± 0.49	4.01 ± 0.45	0	75.21 ± 7.65	84.27 ± 0.85
MDMM + FFC	0.81 ± 0.25	4.84 ± 0.44	4.33 ± 0.65	1.37 ± 1.37	87.72 ± 3.86	96.45 ± 0.81*
(C) MDMD	1.18 ± 0.47	4.51 ± 0.37	6.40 ± 0.23	5.54 ± 1.23	78.48 ± 1.18	82.96 ± 1.23
MDMD + FFC	1.44 ± 0.51	6.40 ± 0.76	15.95 ± 1.44*	5.05 ± 3.38	89.52 ± 1.64*	94.32 ± 1.20*
(D) MDMDF	1.16 ± 0.40	10.43 ± 1.48	20.78 ± 3.46	6.11 ± 3.09	11.53 ± 1.28	13.97 ± 0.36
MDMDF + FFC	1.34 ± 0.49	17.47 ± 5.06	42.37 ± 4.94*	5.92 ± 2.64	19.59 ± 1.10*	23.52 ± 0.44*
(E) MDMD	0.73 ± 0.09	6.23 ± 1.10	9.14 ± 1.76	2.74 ± 1.85	94.19 ± 2.08	98.83 ± 0.42
MDMD + PSC	1.33 ± 0.43	6.20 ± 0.03	8.53 ± 0.56	5.80 ± 0.69	75.88 ± 3.07*	84.57 ± 2.42*
(F) MDMDF	1.04 ± 0.13	14.68 ± 1.55	34.47 ± 6.13	5.51 ± 1.00	17.20 ± 1.65	21.34 ± 1.14
MDMDF + PSC	1.41 ± 0.39	10.42 ± 1.45	33.97 ± 3.21	10.38 ± 0.95*	10.37 ± 0.37*	13.01 ± 2.18*

Epidermal cell suspensions derived from 0.5-day-old mice were cultured in MDM (A), MDMM (B), MDMD (C, E), and MDMDF (D, F) with or without FFC (A–D) or PSC (E, F). Pure melanoblasts or melanocytes were obtained after 14 days. The number of melanoblasts and melanocytes was counted at 1, 7, and 14 days. The percentage of melanocytes in the melanoblast-melanocyte population was also scored. The data are the averages of results from three experiments (mean ± S.E.). Each experiment was performed with different litters of mice. Statistically significant difference ($p^* < 0.05$, underlined).

tion of cultured melanocytes in the presence of keratinocyte-derived factors,[6] it can be determined using this medium whether FFC exhibits proliferation- and differentiation-stimulating activity toward melanocytes in cooperation with DBcAMP. When the epidermal cell suspensions were cultured in MDMD, pigment-producing differentiated melanocytes appeared around keratinocyte colonies within 2–3 days and increased in number. After 14 days, almost all keratinocytes had died and cultures of differentiated melanocytes were obtained (Fig. 2C). In the case of MDMD with FFC, large keratinocyte colonies with numerous enlarged keratinocytes were observed. The number of

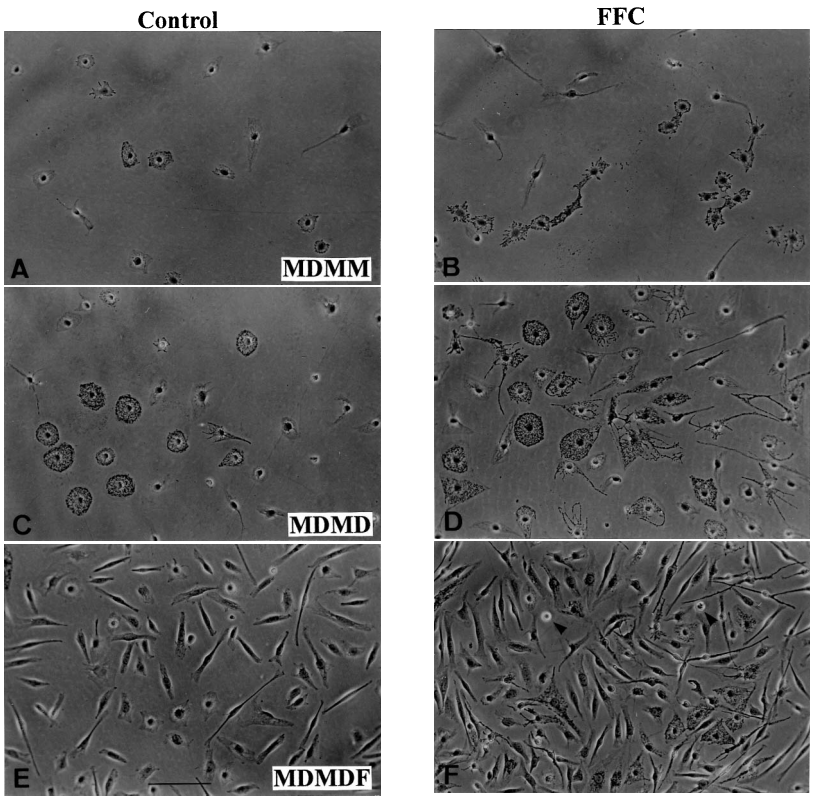

Fig. 2. Effects of FFC on the Proliferation and Differentiation of Melanocytes in MDMM, MDMD, and MDMDF

Epidermal cell suspensions were cultured in MDMM (A) or MDMM with FFC (B). After 14 days, pure culture of melanocytes was obtained (A). In FFC cultures, melanocytes had well-developed dendrites and increased pigmentation (B). Similarly, melanocytes cultured in MDMD with FFC had well-developed dendrites, enlarged cytoplasm, and increased pigmentation (D) compared with control cultures (C). Melanocytes cultured in MDMD with FFC increased dramatically (D). Melanoblasts and melanocytes cultured in MDMDF with FFC increased dramatically (F) compared with control cultures (E). Many mitotic figures (arrowheads) are observed in FFC (F). Phase-contrast microscopy. Bar, 100 μm.

keratinocytes per colony at 2 days doubled (FFC: 17.52 ± 2.65, $n = 3$, control: 6.86 ± 1.22, $n = 3$, $p < 0.05$). The number of melanocytes also doubled (Table 1C). Numerous mitotic melanocytes were observed (Table 2 A). Moreover, melanocyte differentiation was stimulated (Table 1C) and, in addition, dendritogenesis, cell expansion, and pigmentation were increased (Fig. 2D). These results suggest that FFC can stimulate the proliferation, differentiation, dendritogenesis, and pigmentation of melanocytes in cooperation with DBcAMP.

MDMDF containing bFGF and DBcAMP is used for stimulating the proliferation of undifferentiated melanoblasts. Since bFGF in the presence of DBcAMP stimulates melanoblast proliferation in cooperation with keratinocyte-derived factor,[6] this medium can be used to determine whether FFC exhibits proliferation-stimulating activity toward melanoblasts. When the epidermal cell suspensions were cultured in MDMDF, undifferentiated melanoblasts were observed around keratinocyte colonies within 1 day, and increased in number after 3–4 days. After 14 days, almost all the keratinocytes had died and pure cultures of numerous melanoblasts (*ca.* 90%) and a small number of melanocytes (*ca.* 10%) were obtained (Fig. 2E). In the case of MDMDF with FFC, large keratinocyte colonies with numerous enlarged keratinocytes were observed. The number of keratinocytes per colony at 2 days doubled (FFC: 16.63 ± 3.66, $n = 3$; control: 7.49 ± 1.16, $n = 3$, $p < 0.05$). The number of melanoblasts and melanocytes (Fig. 2F and Table 1D) also doubled compared with that in the control cultures (Fig. 2E and Table 1D). Numerous mitotic melanoblasts were observed (Fig. 2F and Table 2B). Moreover, the percentage of differentiated melanocytes in the melanoblast-melanocyte population increased significantly (Table 1D). These results suggest that FFC can stimulate the proliferation of melanoblasts in cooperation with bFGF and DBcAMP.

In this study, FFC stimulated the proliferation and differentiation of mouse epidermal keratinocytes in MDM, MDMM, MDMD, and

Table 2. Mitotic Indices of Melanoblasts and Melanocytes Cultured in MDMD or MDMDF with or without FFC in the Presence or Absence of Keratinocytes

Medium	7 days	14 days	15 days	17 days	21 days
(A) MDMD	0.14 ± 0.14	0	—	—	—
MDMD + FFC	0.78 ± 0.42	0.18 ± 0.04	—	—	—
(B) MDMDF	1.24 ± 0.20	0.18 ± 0.05	—	—	—
MDMDF+ FFC	1.84 ± 0.47	0.41 ± 0.15	—	—	—
(C) MDMD	—	0	0	0	0
MDMD + FFC	—	0	0.15 ± 0.04*	0.20 ± 0.01	0.24 ± 0.11
(D) MDMDF	—	0.18 ± 0.05	0.16 ± 0.07	0.08 ± 0.06	0.05 ± 0.02
MDMDF+ FFC	—	0.24 ± 0.01	0.30 ± 0.03	0.29 ± 0.05	0.25 ± 0.06*

Number of mitotic melanoblasts and melanocytes was counted at 7 and 14 days of primary culture of epidermal cell suspensions in MDMD (A) or MDMDF (B) with or without FFC (A, B). FFC was also added from 14 days (keratinocyte depletion), and mitotic melanoblasts or melanocytes were counted at 14, 15, 17, and 21 days in MDMD (C) or MDMDF (D). Mitotic indices were estimated by calculating the proportion of mitotic melanoblasts and melanocytes to the total number of melanoblasts and melanocytes (%). The data are the averages of results from three experiments mean ± S.E. Each experiment was performed with different litters of mice. Statistically significant difference ($p^* < 0.05$, underlined).

MDMDF. The proliferation of mammalian keratinocytes is regulated by numerous factors including epidermal growth factor,[13] keratinocyte growth factor,[14] and hydrocortisone.[3] It should be emphasized that FFC can stimulate the proliferation of keratinocytes in the absence of these growth factors or hormones. FFC possibly stimulates the proliferation of keratinocytes in the absence of such growth factors or hormones by activating signaling pathways involved in proliferation.

Effects of FFC on the Proliferation and Differentiation of Melanoblasts or Melanocytes in the Absence of Keratinocytes

Primary cultures of epidermal cell suspensions contain keratinocytes in addition to melanoblasts and melanocytes. Therefore it cannot be ascertained whether FFC directly stimulates the proliferation and differentiation of melanoblasts and melanocytes. To investigate whether FFC exhibits proliferation- and differentiation-stimulating activity toward melanocytes in the absence of keratinocytes, pure primary melanoblasts cultured in MDM for 14 days (keratinocyte depletion) were cultured in MDM with or without FFC for another 7 days. Although FFC failed to increase the number of melanoblasts and melanocytes (data not shown), FFC increased the percentage of melanocytes in the melanoblast-melanocyte population from 3% to 39%. These results suggest that FFC can directly stimulate the differentiation from melanoblasts to melanocytes in culture.

To investigate whether FFC stimulates the differentiation of melanocytes in the absence of keratinocytes, pure primary melanocytes cultured in MDMM for 14 days (keratinocyte depletion) were cultured in MDMM with or without FFC for another 7 days. Although FFC failed to increase the number of melanocytes (data not shown), FFC increased the percentage of melanocytes in the melanoblast-melanocyte population from 83% to 97%. Moreover, melanocytes cultured with FFC had increased dendricity and pigmentation. These results suggest that FFC can directly stimulate the differentiation, dendritogenesis, and pigmentation of cultured melanocytes in cooperation with α-MSH.

To investigate whether FFC stimulates the proliferation and differentiation of melanocytes in the absence of keratinocytes, pure primary melanocytes cultured in MDMD for 14 days (keratinocyte depletion) were cultured in MDMD with or without FFC for another 7 days. The number of melanocytes more than doubled (Fig. 3A). Numerous mitotic melanocytes were observed (Table 2C). Moreover, the melanocytes had increased dendricity, enlarged cytoplasms, and increased pigmentation, although control melanocytes showed no change. The percentage of melanocytes in the melanoblast-melanocyte population increased significantly from 88% to 97%. These results suggest that FFC can directly stimulate the proliferation, differentiation, dendritogenesis, and pigmentation of cultured melanocytes in cooperation with DBcAMP.

To investigate whether FFC stimulates the proliferation of melanoblasts in the absence of keratinocytes, pure primary melanoblasts cultured in MDMDF for 14 days (keratinocyte depletion) were cultured in MDMDF with or without FFC for another 7 days. The number of melanoblasts and

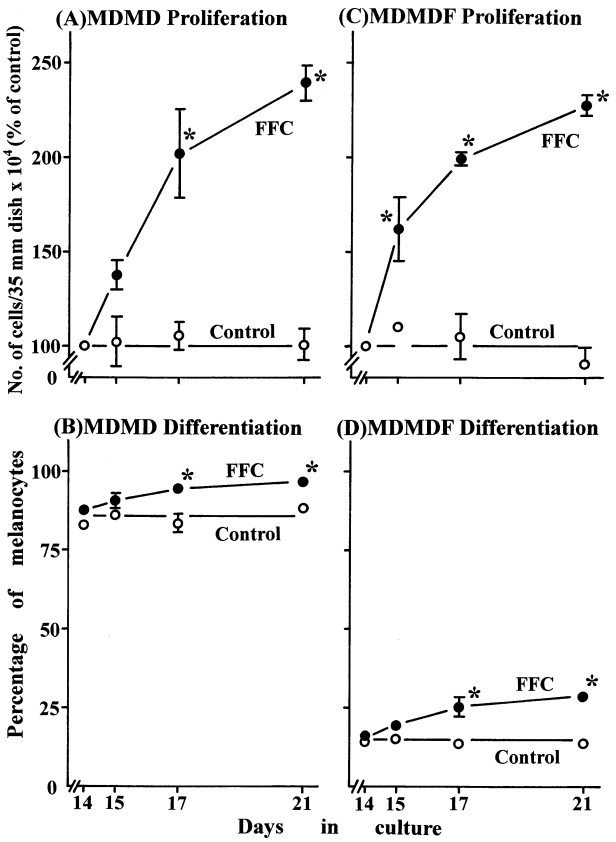

Fig. 3. Kinetics of the Proliferation (A, C) and Differentiation (B, D) of Melanoblasts or Melanocytes Cultured in MDMD with or without FFC (A, B) and in MDMDF with or without FFC (C, D)

Epidermal cell suspensions were cultured in MDMD or MDMDF for 14 days. Pure cultured melanocytes (MDMD) or melanoblasts and melanocytes (MDMDF) were obtained. They were cultured in MDMD or MDMDF with (FFC, •) or without (control, ○) FFC for another 7 days. The number of cells is expressed as percentage of control. The number of melanoblasts and melanocytes was counted and the percentage of melanocytes in the melanoblast-melanocyte population was calculated. The data are the averages of results from three experiments. Each experiment was performed with different litters of mice. Bars indicate standard errors of the mean and are shown only when they were larger than symbols. Statistically significant differences ($p^* < 0.05$).

melanocytes more than doubled (Fig. 3C). Numerous mitotic melanoblasts were observed (Table 2D). Moreover, the percentage of melanocytes in the melanoblast-melanocyte population increased from 14% to 29% (Fig. 3D). These results suggest that FFC can directly stimulate the proliferation of cultured melanoblasts in cooperation with bFGF and DBcAMP.

FFC was revealed to stimulate the proliferation of melanoblasts or melanocytes cultured in MDMD or MDMDF irrespective of the presence or absence of keratinocytes. FFC failed to stimulate the proliferation of melanoblasts or melanocytes in MDM or MDMM. MDM and MDMM do not contain DBcAMP and bFGF, which are indispensable for the proliferation of melanoblasts or melanocytes. Thus FFC appears to be incapable of stimulating the proliferation of melanoblasts or melanocytes in the absence of DBcAMP or bFGF. FFC alone stimulated the differentiation of melanocytes cultured in MDMM or MDMD. It should be emphasized that FFC induced the differentiation of melanocytes in MDM, which is devoid of melanogens, such as α-MSH or DBcAMP, in the absence of keratinocytes. It has been reported that melanocyte proliferation and differentiation can be induced by several factors such as bFGF,[15] endothelin (ET)-1,[16–18] ET-2,[18] ET-3,[18] leukemia inhibitory factor (LIF),[19] Steel factor (SLF or stem cell factor),[20,21] granulocyte-macrophage colony-stimulating factor (GMCSF),[22,23] and hepatocyte growth factor (HGF).[24,25] However, these growth factors or cytokines require the presence of bFGF or adenosine 3′:5′-cyclic monophosphate-elevating agents such as DBcAMP to stimulate the proliferation and differentiation of melanoblasts or melanocytes.[26] It should be emphasized that FFC can stimulate the proliferation of melanoblasts and melanocytes and, in addition, induce the differentiation of melanocytes in the absence of these growth factors or cytokines. Stimulation of the proliferation and differentiation of mammalian melanocytes is elicited by protein kinase C (PKC) activated by ET-1, ET-2, or ET-3 as well as mitogen-activated protein kinase activated by bFGF, SLF, LIF, GMCSF, or HGF in addition to protein kinase A (PKA) activated by α-MSH or DBcAMP.[26] PKA increases the activity of TYR, TRP-1, and TRP-2 and increases melanin synthesis by these enzymes.[19,26–30] FFC is assumed to activate the signaling pathways of PKC, MAP kinase, and PKA, even in the absence of growth factors and cytokines, or to stimulate the proliferation and differentiation of melanoblasts and melanocytes.

Effects of PSC on the Proliferation and Differentiation of Keratinocytes and Melanoblasts or Melanocytes

Since PSC-DDW contains several minerals, their effects on the proliferation and differentiation of keratinocytes and melanoblasts or melanocytes in the primary culture can be investigated. Since similar minerals in addition to FFC are included in FFC-DDW, the effects of such minerals included in FFC-DDW on the proliferation and differentiation of keratinocytes and melanoblasts or

melanocytes may be investigated using PSC-DDW. When the epidermal cell suspensions of newborn mouse skin were cultured in MDMD with PSC-DDW, large keratinocyte colonies with numerous extended keratinocytes were observed. The number of keratinocytes per colony at 2 days doubled (PSC: 15.27 ± 2.77, $n = 3$; control: 6.86 ± 1.22, $n = 3$, $p < 0.05$) like those cultured with FFC. However, the number of melanocytes failed to increase (Table 1E). The number of mitotic melanocytes did not differ between the control and PSC-DDW (data not shown). In contrast, the percentage of melanocytes in the melanoblast-melanocyte population significantly decreased (Table 1E). Large keratinocyte colonies with numerous extended keratinocytes were similarly observed in MDMDF with PSC-DDW. The number of keratinocytes per colony at 2 days doubled (PSC: 15.30 ± 2.64, $n = 3$; control: 7.49 ± 1.16, $n = 3$, $p < 0.05$). However, the number of melanoblasts and melanocytes failed to increase (Table 1F). The number of mitotic melanoblasts did not differ between the control and PSC-DDW (data not shown). In contrast, the percentage of melanocytes in the melanoblast-melanocyte population at 7 and 14 days decreased (Table 1F). These results suggest that PSC stimulates the proliferation and differentiation of keratinocytes in a fashion similar to FFC. The several minerals included in PSC-DDW appear to stimulate the proliferation and differentiation of keratinocytes in the absence of growth factors or hormones. However, PSC failed to stimulate the proliferation and differentiation of melanoblasts or melanocytes, possibly due to the absence of FFC in PSC-DDW. Therefore it is conceivable that either FFC or minerals can cause the proliferation and differentiation of keratinocytes, whereas the proliferation and differentiation of melanoblasts or melanocytes require the presence of FFC in addition to minerals.

In this study, the extent of the stimulation of keratinocyte proliferation by FFC was similar to that of melanoblast or melanocyte proliferation (*ca.* two-fold increase), suggesting that the proliferation of the two types of cells constituting the epidermis may be equally stimulated by FFC and the homeostasis of epidermal components may be controlled by FFC. In addition to the stimulation of the proliferation of keratinocytes and melanoblasts or melanocytes, FFC stimulated the differentiation of keratinocytes and melanocytes, suggesting that the renewal of cells constituting the epidermis may be accelerated by FFC. Thus FFC is assumed to make the skin healthy by exchanging old keratinocytes and melanoblasts or melanocytes for new ones. Further study using *in vivo* test systems to confirm the proliferation- and differentiation-stimulating effects of FFC on keratinocytes and melanoblasts or melanocytes remains to be performed.

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