Ferrous Ferric Chloride Induces the Differentiation of Cultured Mouse Epidermal Melanocytes Additionally with Herbal Medicines

Tomohisa Hirobe*

Radiation Effect Mechanism Research Group, National Institute of Radiological Sciences, 4–9–1, Anagawa, Inage-ku, Chiba 263–8555, Japan and Graduate School of Science, Chiba University, 1–33, Yayoi-cho, Inage-ku, Chiba 263–8522, Japan

(Received August 7, 2008; Accepted October 31, 2008; Published online November 5, 2008)

Ferrous ferric chloride (FFC) is a special form of aqueous iron that is a complex of ferrous chloride and ferric chloride and participates in oxidation and reduction reactions. My previous study showed that FFC stimulated the proliferation and differentiation of cultured epidermal melanoblasts or melanocytes derived from newborn mice. However, it is not known whether FFC stimulates the proliferation and differentiation of melanocytes in cooperation with natural factors, such as vinegars, vitamins, and herbal medicines. The drink Pairogen® (Akatsuka Co., Tsu, Japan) consists of FFC, vinegars, and vitamins, while Pairogen Gold® (Akatsuka Co.) contains herbal medicines in addition to FFC, vinegar, and vitamins. To clarify whether these natural factors supplemented to Pairogen or Pairogen Gold elicit stimulative effects on skin function, Pairogen and Pairogen Gold were added to the culture medium and tested for their proliferation- and differentiation-stimulating activity on melanocytes. Although Pairogen Gold failed to increase melanocyte proliferation beyond that elicited by FFC alone, it markedly increased melanocyte differentiation. In contrast, Pairogen possessed no such effect. The extracts of Chinese wolfberry (*Lycium chinense*) and Siberian ginseng (*Eleutherococcus senticosus*) that are included in Pairogen Gold stimulated melanocyte differentiation additionally with FFC. Therefore, these results suggest that FFC is involved in regulating the differentiation of melanocytes additionally with extracts of Chinese wolfberry and Siberian ginseng.

Key words —— melanoblast, melanocyte, ferrous ferric chloride, proliferation, differentiation, herbal medicine

INTRODUCTION

Ferrous ferric chloride (FFC®, Akatsuka Co., Tsu, Japan) is a special form of aqueous iron that is a complex of ferrous chloride and ferric chloride and participates in oxidation and reduction reactions.[1)] FFC possesses a specific biological effect such as stimulation of the growth of plants, especially root growth.[2)] In animals, FFC also possesses a stimulative effect on cell growth.[3)] My previous study showed that FFC stimulated the proliferation of cultured keratinocytes and melanoblasts or melanocytes derived from newborn mice.[3)] FFC also stimulated the differentiation of keratinocytes and melanocytes.[3)] The proliferation of keratinocytes and 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 mouse epidermis may be equally stimulated by FFC.[3)] Thus, FFC is thought to activate skin function by promoting cell renewal via the stimulation of the proliferation and differentiation of keratinocytes and melanoblasts or melanocytes.[3)] However, it is not known whether FFC can stimulate the proliferation and differentiation of skin cells in cooperation with natural factors such as vinegars, vitamins, and herbal medicines.

Pairogen® (Akatsuka Co.) is a refreshing drink, 90% of which consists of FFC water and the remaining 10% of other ingredients (purified, not artificially synthesized), such as rice vinegar, Japanese apricot vinegar, apple vinegar, persimmon vinegar, vitamins B_2, B_6, and C, glucose, fructose, citric acid, and malic acid. Pairogen Gold® (Akatsuka Co.) is another refreshing drink consisting of 90% FFC water; the remaining 10% consists of other ingredients (purified, not artificially synthesized), such as apple vinegar, vitamins B_1, B_2, B_6 and C, glucose, fructose, citric acid, malic

*To whom correspondence should be addressed: Radiation Effect Mechanism Research Group, National Institute of Radiological Sciences, 4–9–1, Anagawa, Inage-ku, Chiba 263–8555, Japan. Tel.: +81-43-206-3253; Fax: +81-43-206-4638; E-mail: thirobe@nirs.go.jp

acid, polydextrose, and extracts of Korean ginseng (*Panax ginseng*), Chinese wolfberry (*Lycium chinense*), and Siberian ginseng (*Eleutherococcus senticosus*). Pairogen Gold is known to be more effective for maintaining a healthy condition of internal organs, such as intestines, than Pairogen. Pairogen Gold is also known to be more effective for making skin healthy. These effects of Pairogen Gold might be elicited by the combined effects of FFC and factors supplemented in Pairogen Gold, since the content of FFC does not differ between Pairogen and Pairogen Gold. It is possible that the herbal medicines that are included in Pairogen Gold, but not in Pairogen may be responsible for the stimulative effects of Pairogen Gold on skin function. Thus, the question arises as to which herbal medicines supplemented in Pairogen Gold are responsible for the stimulative effects on skin function. In this study, to solve this problem, *in vitro* culture systems[4,5] of mouse keratinocytes and melanocytes were used. FFC-containing Pairogen or Pairogen Gold was added to the culture medium and their proliferation- and differentiation-stimulating effects on skin cells were studied using a serum-free culture system of primary keratinocytes[4] and melanoblasts or melanocytes[5] derived from the skin of newborn C57BL/10JHir mice.

MATERIALS AND METHODS

Mice —— Strain C57BL/10JHir mice (black, house mouse) were provided with water and a commercial diet, OA-2 (Clea Japan, Tokyo, Japan), *ad libitum*. They were housed in individual cages, 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 used for obtaining epidermal cell suspensions from dorsal skins of 0.5-day-old mice was reported previously.[6] In brief, disaggregated epidermal cell suspensions were pelleted by centrifugation and suspended in Ham's F-10 medium. After centrifugation, cell pellets were resuspended in the culture medium.[7] 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 for all experiments in this study. The cells in epidermal cell suspensions 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 fresh medium four times a week. After 14 days, almost pure cultures of melanoblasts or melanocytes were obtained.

FFC can be prepared as follows.[1] Ferric chloride ($FeCl_3 \cdot 6H_2O$) 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. An additional 1 g of $FeCl_3 \cdot 6H_2O$ per 10 ml of solution is dissolved in the solution, which is then gradually heated to 100°C until FFC can be crystallized.[1] The proportion of Fe(II) to Fe(III) is 4 : 6 (10^{-8} mM solution), 6 : 4 (10^{-12} mM solution), and 7 : 3 (10^{-14} mM solution).[1] Analysis using ion-exchange chromatography and X-ray diffractometry suggests that Fe(II) and Fe(III) form dimers.[1] Water containing FFC can be obtained by immersing FFC Ceramic Beads® (Akatsuka Co.) in water. These Beads are prepared by heating hardened soil with FFC solution to approximately 800°C. FFC Ceramic Beads® are made of porous ceramic treated with FFC solution. One day prior to initiation of the culture, 60 g of FFC Ceramic Beads® was immersed in 3 l of deionized and distilled water (DDW) for 24 hr in a glass bottle in a styrene foam box at room temperature. This DDW is referred to here as FFC-DDW (20 g/l). In FFC-DDW, various minerals such as Ca, Mg, K, Na, Fe, Cu,

etc., are present in addition to FFC.[8] Powdered F-10 medium was dissolved in DDW and FFC-DDW, then MDM, MDMM, MDMD, and MDMDF with or without FFC were prepared. In some experiments, Pairogen and Pairogen Gold, at dilutions of 10^{-2}, 10^{-3}, and 10^{-4}, were added to MDM, MDMM, MDMD, and MDMDF prepared from DDW 1 day prior to intiation of the primary culture, and the media were stored at 2°C. The effects of FFC and the extracts of herbal medicines (extracted by ethanol, generously supplied by Akatsuka Co.) that are included in Pairogen Gold on the proliferation and differentiation of keratinocytes and melanoblasts or melanocytes were also examined. The same concentrations of the extracts of herbal medicines that are included in Pairogen Gold were added to the culture medium containing FFC.

Assays for Proliferation and 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 statistical significance of differences in the number of melanoblasts and melanocytes and in the percentage of melanocytes in the melanoblast-melanocyte population were determined between controls and experiments using Student's *t*-test for comparison of groups of equal size.

Bipolar, tripolar, dendritic, polygonal, or epithelioid cells, as seen by phase-contrast microscopy, which contained brown or black pigment granules, as observed by bright-field microscopy, were scored as differentiated melanocytes (pigmented melanocytes). The number of differentiated melanocytes was similar to the number of L-3,4-dihydroxyphenylalanine (dopa)-positive melanocytes.[9] In contrast, bipolar, tripolar, dendritic, or polygonal cells, as seen by phase-contrast microscopy, which contained no pigment and were negative for dopa staining, as observed by bright-field microscopy, were scored as melanoblasts. These cells were stained with the combined dopa-premelanin reaction (combined dopa-ammoniacal silver nitrate staining).[10,11] This staining preferentially reveals undifferentiated melanoblasts that contain unmelanized stage I and II melanosomes in addition to tyrosinase-containing differentiated melanocytes. The ammoniacal silver nitrate reaction specifically reveals unmelanized stage I and II melanosomes as well as melanized stage III and IV melanosomes in melanocytes, and the metallic silver particles are deposited with a high degree of selectivity.[12,13] Melanoblasts were also stained with antibodies to tyrosinase-related protein (TRP)-1 and TRP-2 (or dopachrome tautomerase).[14] A melanoblast was defined here as an unpigmented cell that had no tyrosinase activity. Melanoblasts cultured in MDMM, MDMD, or MDMDF were stained using the combined dopa-premelanin reaction as well as with antibodies to TRP-1 and TRP-2.[14] However, melanoblasts cultured in MDM were negative to the combined dopa-premelanin reaction as well as to TRP-1 and TRP-2.[14]

RESULTS

Effects of FFC, Pairogen, and Pairogen Gold on Proliferation and Differentiation of Melanoblasts

MDM is used to define and maintain melanoblasts. Since MDM does not contain melanogens such as α-MSH or DBcAMP,[5–7] this medium can be used to determine whether FFC, Pairogen, or Pairogen Gold possesses differentiation-stimulating activity for melanoblasts. Bipolar, tripolar, or dendritic melanoblasts were in contact with adjacent keratinocyte colonies through a dendritic process within a day or two after initiation of the primary culture. After 3 days, the melanoblasts gradually increased in number. After 7–8 days, the keratinocyte colonies gradually decreased, and by 14 days, almost all keratinocytes had died and pure cultures of melanoblasts (Fig. 1A) were obtained. In case of MDM with FFC, Pairogen, or Pairogen Gold, large keratinocyte colonies containing numerous enlarged keratinocytes were observed. Keratinocyte proliferation and differentiation were stimulated similarly in all groups. Although the number of melanoblasts failed to increase in MDM with FFC (Fig. 1B, Table 1IA), Pairogen (Fig. 1C, Table 1IIA), and Pairogen Gold (Fig. 1D, Table 1IIIA), numerous differentiated melanocytes (Fig. 1B–1D) were observed. Moreover, there was an increase in dendritogenesis, cell expansion, and pigmentation of melanocytes (Fig. 1B–1D). Stimulation of melanocyte differentiation was greatest for Pairogen Gold (Table 1IIIA). At dilution of 10^{-2}, more than 90% of cells were differentiated melanocytes (Table 1IIIA). The differentiation-

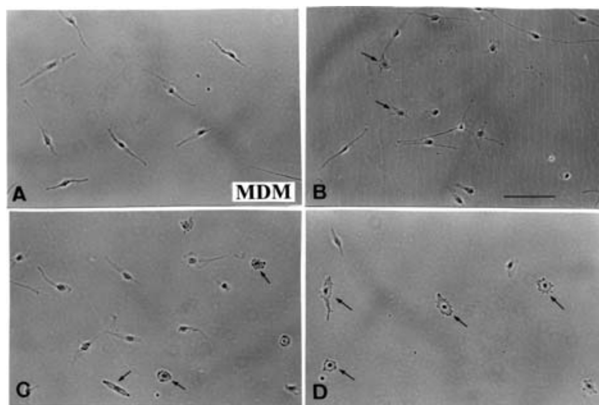

Fig. 1. Effects of FFC, Pairogen and Pairogen Gold on Proliferation and Differentiation of Mouse Epidermal Melanoblasts in MDM

Epidermal cell suspensions derived from mouse skin were cultured in MDM (A) or in MDM with FFC (B, 20 g/l), Pairogen (C, dilution of 10^{-2}), or Pairogen Gold (D, dilution of 10^{-2}). After 14 days, pure cultures of melanoblasts were obtained in control culture (A). However, in the cultures with FFC (B), Pairogen (C), and Pairogen Gold (D), many differentiated melanocytes (arrows) were observed. Pairogen Gold (D) was most effective. Phase-contrast microscopy. Bar, 100 μm.

stimulating effect of FFC (20 g/l, Table 1IA) was similar to that of Pairogen at 10^{-2} dilution (Table 1IIA) and to that of Pairogen Gold at 10^{-3} dilution (Table 1IIIA). The differentiation-stimulating effect of Pairogen Gold was approximately 10-fold higher than that of Pairogen.

Effects of Herbal Medicines on Differentiation of Melanocytes

Since the differentiation-stimulating activity of Pairogen did not differ from that of FFC, the presence of vinegars and vitamins in Pairogen did not appear to enhance the melanocyte differentiation effect of FFC. In contrast, the differentiation-stimulating effect of Pairogen Gold markedly exceeded that of FFC, suggesting that the herbal medicines included in Pairogen Gold possess additional stimulating effects on melanocyte differentiation induced by FFC. To clarify which herbal medicines are concerned, extracts of Korean ginseng, Chinese wolfberry, or Siberian ginseng were added to MDM with or without FFC. Addition of any one of the three kinds of extract to FFC, or of either of the combinations of Korean ginseng plus Chinese wolfberry or Korean ginseng plus Siberian ginseng with FFC failed to increase the degree of melanocyte differentiation induced. In contrast, the combination of Chinese wolfberry and Siberian ginseng with FFC markedly increased the percentage of melanocytes to a level similar to that obtained with Pairogen Gold (Fig. 2). Three combinations with FFC showed a level of melanocyte differentiation similar to that induced by the two combinations of Chinese wolfberry and Siberian ginseng with FFC (Fig. 2). The percentages of melanocytes obtained with Pairogen Gold, Chinese wolfberry plus Siberian ginseng, and Korean ginseng plus Chinese wolfberry plus Siberian ginseng were significantly greater than those obtained with FFC alone (Fig. 2, $p < 0.05$). In contrast, single treatment, two and three combinations without FFC failed to exceed the degree of melanocyte differentiation induced by FFC alone (Fig. 2).

Effects of Pairogen and Pairogen Gold on Proliferation and Differentiation of Melanoblasts and Melanocytes

MDMM containing α-MSH is used to stimulate melanocyte differentiation. Since α-MSH is known to stimulate melanocyte differentiation *in vivo* and *in vitro*,[7] this medium can be used to determine whether Pairogen or Pairogen Gold stimulates differentiation-stimulating activity toward melanoblasts in cooperation with α-MSH. When epidermal cell suspensions were cultured in MDMM, pigment-producing differentiated melanocytes appeared around keratinocyte colonies within 2–3 days and then increased in number. After 14 days, almost all the keratinocytes had died and pure cultures of differentiated melanocytes were obtained (Fig. 3A). In case of MDMM with Pairogen or Pairogen Gold, large keratinocyte colonies with numerous enlarged keratinocytes were observed, similar to those obtained in cultures of MDM with FFC, suggesting that Pairogen or Pairogen Gold stimulates keratinocyte proliferation and differentiation. Although the number of melanoblasts and melanocytes failed to increase (Table 1IIB, 1IIIB) in culture with Pairogen or Pairogen Gold (Fig. 3B), melanocyte differentiation was stimulated (Fig. 3B, Table 1IIB, 1IIIB), and dendritogenesis, cell expansion, and pigmentation also increased (Fig. 3B).

MDMD containing DBcAMP is used to stimulate both the proliferation and differentiation of melanocytes. Since DBcAMP is known to stimulate both proliferation and differentiation of cultured melanocytes in the presence of keratinocyte-derived factors,[4] this medium can be used to determine whether Pairogen or Pairogen Gold stimulates proliferation and differentiation activity toward melanocytes in cooperation with DBcAMP. When epidermal cell suspensions were

Table 1. Effects of FFC, Pairogen, and Pairogen Gold on Proliferation and Differentiation of Mouse Epidermal Melanoblasts and Melanocytes

Medium (dilution)	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
(I) FFC						
(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 + 20 g/l	0.40 ± 0.05	3.69 ± 0.62	3.94 ± 0.47	2.56 ± 1.31	27.19 ± 6.44*	43.69 ± 1.65*
(II) Pairogen						
(A) MDM	0.38 ± 0.06	2.69 ± 0.56	2.75 ± 0.71	0.28 ± 0.28	1.38 ± 1.14	4.47 ± 0.90
MDM + 10^{-4}	0.44 ± 0.08	3.17 ± 0.35	2.90 ± 0.81	0.46 ± 0.46	7.04 ± 2.75	14.87 ± 3.04*
MDM + 10^{-3}	0.45 ± 0.13	2.62 ± 0.69	2.57 ± 0.74	1.48 ± 1.48	13.92 ± 2.97*	15.53 ± 1.29*
MDM + 10^{-2}	0.35 ± 0.04	2.54 ± 0.88	2.41 ± 0.72	0.80 ± 0.80	30.67 ± 3.55*	33.87 ± 4.73*
(B) MDMM	0.54 ± 0.03	3.11 ± 0.78	4.23 ± 0.91	0.73 ± 0.73	76.76 ± 4.36	88.59 ± 2.50
MDMM + 10^{-4}	0.73 ± 0.11	4.08 ± 0.91	4.10 ± 0.91	1.58 ± 0.86	89.67 ± 3.23	91.71 ± 2.31
MDMM + 10^{-3}	0.69 ± 0.07	3.32 ± 0.80	3.39 ± 0.31	2.08 ± 1.11	91.43 ± 2.94*	96.94 ± 0.94*
MDMM + 10^{-2}	0.67 ± 0.01	3.15 ± 0.66	3.46 ± 0.22	2.80 ± 1.73	93.86 ± 1.12*	96.51 ± 1.76*
(C) MDMD	1.11 ± 0.22	6.07 ± 0.21	7.90 ± 1.11	1.83 ± 1.28	83.29 ± 6.06	91.99 ± 0.96
MDMD + 10^{-4}	1.08 ± 0.22	7.52 ± 1.72	13.01 ± 3.22*	3.02 ± 1.82	91.32 ± 3.47	95.39 ± 1.17
MDMD + 10^{-3}	0.96 ± 0.08	6.65 ± 1.17	9.08 ± 1.59	4.77 ± 3.28	94.08 ± 1.57	97.78 ± 0.37*
MDMD + 10^{-2}	1.12 ± 0.34	8.30 ± 1.11	12.49 ± 2.29	5.74 ± 2.84	95.85 ± 0.90	98.88 ± 0.52*
(D) MDMDF	1.03 ± 0.17	12.10 ± 0.85	30.12 ± 7.71	2.72 ± 1.45	12.90 ± 4.33	13.22 ± 5.30
MDMDF + 10^{-4}	1.11 ± 0.20	22.37 ± 3.27*	58.05 ± 3.21*	6.47 ± 2.42	17.82 ± 4.80	21.82 ± 5.85
MDMDF + 10^{-3}	1.17 ± 0.06	18.16 ± 2.76	41.89 ± 6.20	2.99 ± 1.55	15.09 ± 3.08	15.42 ± 4.46
MDMDF + 10^{-2}	1.44 ± 0.14	21.64 ± 1.04*	44.37 ± 2.66	4.83 ± 2.10	17.86 ± 2.31	21.30 ± 4.88
(III) Pairogen Gold						
(A) MDM	0.35 ± 0.01	3.12 ± 0.37	3.09 ± 0.12	1.52 ± 1.52	2.18 ± 0.66	3.42 ± 0.55
MDM + 10^{-4}	0.49 ± 0.12	2.89 ± 0.36	3.22 ± 0.31	3.88 ± 3.88	14.82 ± 1.46*	20.27 ± 5.09*
MDM + 10^{-3}	0.44 ± 0.13	2.35 ± 0.35	2.71 ± 0.65	3.17 ± 3.17	26.45 ± 6.31*	38.78 ± 10.62*
MDM + 10^{-2}	0.39 ± 0.13	1.81 ± 0.30	1.85 ± 0.44	3.42 ± 3.42	65.30 ± 8.28*	90.19 ± 2.61*
(B) MDMM	0.48 ± 0.19	2.86 ± 0.72	2.59 ± 0.30	0.68 ± 0.68	63.94 ± 4.07	74.30 ± 0.57
MDMM + 10^{-4}	0.67 ± 0.14	3.26 ± 0.49	2.71 ± 0.62	3.51 ± 3.51	82.05 ± 1.89*	88.71 ± 1.40*
MDMM + 10^{-3}	0.57 ± 0.16	2.33 ± 0.23	2.62 ± 0.11	5.57 ± 2.80	82.05 ± 1.56*	92.59 ± 2.42*
MDMM + 10^{-2}	0.64 ± 0.13	2.01 ± 0.53	1.86 ± 0.49	6.31 ± 4.32	93.54 ± 0.67*	98.77 ± 0.65*
(C) MDMD	0.88 ± 0.26	4.05 ± 0.85	6.29 ± 0.81	3.16 ± 1.69	74.14 ± 2.15	86.22 ± 0.82
MDMD + 10^{-4}	1.06 ± 0.18	6.92 ± 1.25	15.05 ± 1.32*	2.87 ± 1.02	90.90 ± 3.01*	96.83 ± 1.01*
MDMD + 10^{-3}	1.36 ± 0.30	6.36 ± 0.60*	12.84 ± 2.09*	2.61 ± 1.49	91.27 ± 1.91*	96.77 ± 0.66*
MDMD + 10^{-2}	0.87 ± 0.26	3.80 ± 0.84	4.98 ± 1.11	11.43 ± 3.21	96.82 ± 1.27*	99.09 ± 0.31*
(D) MDMDF	0.86 ± 0.32	9.19 ± 1.24	20.32 ± 0.44	4.93 ± 2.95	8.39 ± 1.95	8.30 ± 0.31
MDMDF + 10^{-4}	0.94 ± 0.14	15.03 ± 1.73	40.42 ± 3.64*	4.53 ± 1.84	14.31 ± 3.55	16.54 ± 2.43*
MDMDF + 10^{-3}	0.89 ± 0.09	13.99 ± 2.21	39.49 ± 4.23*	6.38 ± 1.56	14.39 ± 2.89	16.92 ± 1.22*
MDMDF + 10^{-2}	1.05 ± 0.17	12.35 ± 1.12	33.89 ± 3.94*	5.15 ± 1.47	18.33 ± 3.40	14.15 ± 0.36*

Epidermal cell suspensions derived from 0.5-day-old mice were cultured in MDM (A), MDMM (B), MDMD (C), and MDMDF (D) with or without FFC (I), Pairogen (II), or Pairogen Gold (III) at a concentration of 20 g/l (FFC) or dilutions of 10^{-4}, 10^{-3}, and 10^{-2} (Pairogen, Pairogen Gold). 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 (± S.E.M.). Each experiment was performed with different litters of mice. *Statistically significant difference ($p < 0.05$).

cultured in MDMD, pigment-producing differentiated melanocytes appeared around keratinocyte colonies within 2–3 days and then increased in number. After 14 days, almost all keratinocytes died and pure cultures of differentiated melanocytes were obtained (Fig. 3C). In case of MDMD with Pairogen or Pairogen Gold, large keratinocyte colonies with numerous enlarged keratinocytes were observed, similar to those obtained in cultures in MDM with FFC, suggesting that Pairogen or Pairogen Gold stimulates keratinocyte proliferation and differentiation. The number of keratinocytes per colony in 2 days

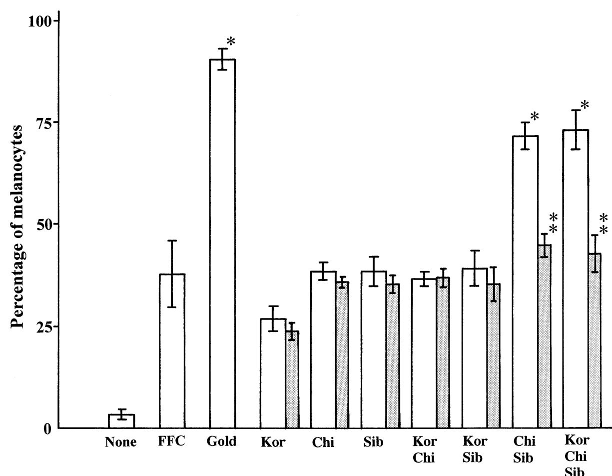

Fig. 2. Effects of FFC, Pairogen Gold, Korean Ginseng (Kor), Chinese Wolfberry (Chi), or Siberian Ginseng (Sib) Extracts with or without FFC on Differentiation of Melanoblasts into Melanocytes in MDM

Epidermal cell suspensions were cultured in MDM (Control). They were also cultured in MDM with FFC (20 g/l), Pairogen Gold (10^{-2} dilution), or any one of the three kinds of extract, either of the two combination, or three combinations with (open box) or without (shadowed box) FFC. After 14 days, pure culture of melanoblasts and melanocytes was obtained. 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 S.E.M. *Statistically significant differences from FFC alone ($p < 0.05$). **Statistically significant difference between Chi + Sib + FFC and Chi + Sib or between Kor + Chi + Sib + FFC and Kor + Chi + Sib ($p < 0.05$).

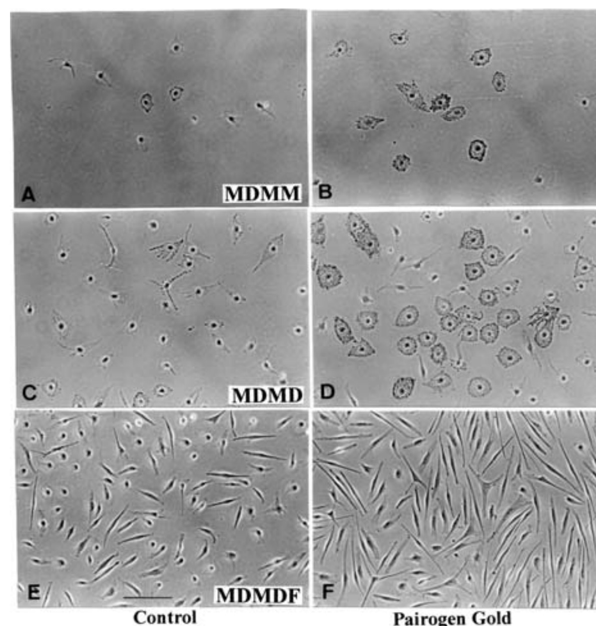

Fig. 3. Effects of Pairogen Gold on Proliferation and Differentiation of Mouse Epidermal Melanoblasts or Melanocytes in MDMM, MDMD, and MDMDF

Epidermal cell suspensions derived from mouse skin were cultured in MDMM (A) or in MDMM with Pairogen Gold at 10^{-2} dilution (B), or in MDMD (C) or in MDMD with Pairogen Gold at 10^{-4} dilution (D), or in MDMDF (E) or in MDMDF with Pairogen Gold at 10^{-4} dilution (F). After 14 days, pure cultures of melanocytes (MDMM and MDMD) were obtained. In the cultures in MDMM or MDMD added with Pairogen Gold at 10^{-2} or 10^{-4} dilution, dendritogenesis, cell expansion and pigmentation of melanocytes increased (B, D). After 14 days, pure cultures of melanoblasts (90%) and melanocytes (10%) were obtained in MDMDF (E). In the culture in MDMDF added with Pairogen Gold at 10^{-4} dilution, many undifferentiated melanoblasts were observed (F). Phase-contrast microscopy. Bar, 100 μm.

doubled in both Pairogen and Pairogen Gold (data not shown). The number of melanocytes also doubled in both Pairogen (Table 1IIC) and Pairogen Gold (Fig. 3D, Table 1IIIC). The most preferable dilution was 10^{-4} for both Pairogen and Pairogen Gold. Numerous mitotic melanocytes were observed. Moreover, melanocyte differentiation was stimulated (Fig. 3D, Table 1IIC, 1IIIC) and dendritogenesis, cell expansion, and pigmentation also increased (Fig. 3D).

MDMDF containing bFGF and DBcAMP is used to stimulate the proliferation of undifferentiated melanoblasts.[5] Since bFGF in the presence of DBcAMP stimulates melanoblast proliferation synergistically with keratinocyte-derived factor,[5] this medium can be used to determine whether Pairogen or Pairogen Gold stimulates the proliferation toward melanoblasts in cooperation with bFGF and DBcAMP. When 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 died and pure cultures of numerous melanoblasts (approximately 90%) and a small number of melanocytes (approximately 10%) were obtained (Fig. 3E). In case of MDMDF with Pairogen or Pairogen Gold, large keratinocyte colonies with numerous enlarged keratinocytes were observed, similar to those observed in cultures of MDM with FFC, suggesting that Pairogen or Pairogen Gold stimulates keratinocyte proliferation and differentiation. The number of keratinocytes per colony on day 2 doubled in both Pairogen and Pairogen Gold (data not shown). The number of melanoblasts and melanocytes also doubled in both Pairogen (Table 1IID) and Pairogen Gold (Fig. 3F, Table 1IIID). The most preferable dilution was 10^{-4} for both Pairogen and Pairogen Gold. Although the percentage of melanocytes failed to increase significantly with Pairogen (Table 1IID), that of melanocytes with Pairogen Gold (Table 1IIID) significantly increased at all dilutions

tested ($p < 0.05$). The percentage of differentiated melanocytes at a dilution of 10^{-4} of Pairogen Gold was slightly lower than that at dilutions of 10^{-2} and 10^{-3} (Table 1IIIC, D). This might be resulted from the increased proliferation of melanoblasts at a dilution of 10^{-4}. These results suggest that Pairogen and Pairogen Gold can stimulate the proliferation of melanoblasts in cooperation with bFGF and DBcAMP.

DISCUSSION

In my previous study, FFC was shown to increase the proliferation of melanoblasts or melanocytes cultured in MDMDF or MDMD by two-fold irrespective of the presence or absence of keratinocytes.[3] FFC failed to stimulate the proliferation of melanoblasts or melanocytes in MDM or MDMM.[3] FFC was also shown to stimulate the differentiation, dendritogenesis, cell expansion, and pigmentation of melanocytes. In the present study, Pairogen and Pairogen Gold similarly stimulated the proliferation of mouse epidermal melanoblasts or melanocytes in MDMDF or MDMD. They also stimulated the differentiation, dendritogenesis, cell expansion, and pigmentation of melanocytes in MDMD. Thus, Pairogen and Pairogen Gold can stimulate the proliferation, differentiation, dendritogenesis, cell expansion, and pigmentation of melanocytes in cooperation with DBcAMP. These results suggest that the presence of vinegars, vitamins, and herbal medicines in Pairogen or Pairogen Gold elicit no additional effect on the proliferation of mouse epidermal melanoblasts or melanocytes. FFC seems to be mainly involved in regulating the proliferation of mouse epidermal melanoblasts or melanocytes in neonatal skin.

In my previous study,[3] FFC induced the differentiation of melanocytes in MDM, which is devoid of melanogens, such as α-MSH or DBcAMP.[5,6] In the present study, Pairogen elicited no additional stimulation of melanocyte differentiation in MDM induced by FFC. In contrast, Pairogen Gold elicited additional stimulation of melanocyte differentiation in MDM. Combined treatment of FFC with extracts of Chinese wolfberry and Siberian ginseng markedly increased the differentiation, dendritogenesis, cell expansion, and pigmentation of melanocytes to a level similar to that elicited by Pairogen Gold. Extracts of Chinese wolfberry and Siberian ginseng stimulated the differentiation of melanocytes in the absence of FFC. These results suggest that FFC and factors present in extracts of Chinese wolfberry and Siberian ginseng additionally induce the differentiation of mouse epidermal melanocytes even in the absence of natural melanogens such as α-MSH or DBcAMP.[5,6]

Although extracts of Korean ginseng,[15] Chinese wolfberry,[16,17] and Siberian ginseng[18–23] are reported to affect the functions of various cells, they have not been reported to stimulate differentiation, dendritogenesis, cell expansion, and pigmentation of melanocytes. This is the first report on the extracts of Chinese wolfberry and Siberian ginseng that stimulate differentiation, dendritogenesis, cell expansion, and pigmentation of melanocytes additionally with FFC. However, it remains to be established what factors present in Chinese wolfberry and Siberian ginseng are involved in regulating the differentiation of mouse epidermal melanocytes additionally with FFC. Syringin, an active principle of Siberian ginseng, stimulates the secretion of β-endorphin in rats.[20] However, it is not yet known whether syringin is involved in regulating the differentiation of melanocytes additionally with Chinese wolfberry-derived factors and FFC.

Stimulation of the differentiation of mammalian melanocytes is elicited by protein kinase C (PKC)[24] activated by endothelin (ET)-1,[25–27] ET-2,[27] or ET-3;[27] by mitogen-activated protein kinase (MAP kinase)[28] activated by bFGF,[29] steel factor (SLF),[30,31] leukemia inhibitory factor (LIF),[32] granulocyte-macrophage colony-stimulating factor (GMCSF),[33,34] or hepatocyte growth factor (HGF);[35,36] and by protein kinase A (PKA)[7,37] activated by α-MSH or DBcAMP.[5,6,37] PKA increases the activity of tyrosinase, TRP-1, and TRP-2 and increases melanin synthesis by these enzymes.[24,37,38] It remains to be investigated by a future study whether extracts of Chinese wolfberry and Siberian ginseng activate the signaling pathways of PKC, MAP kinase, and PKA in cooperation with FFC, even in the absence of melanogens such as α-MSH and DBcAMP.[5,6,37]

In the present study, Pairogen and Pairogen Gold were shown to stimulate the proliferation of melanoblasts or melanocytes in a way similar to FFC,[3] as reported previously. These results suggest that the ingredients of Pairogen and Pairogen Gold, such as vinegars, vitamins, and herbal medicines elicit no increase in the proliferation of melanoblasts or melanocytes elicited by FFC.[3] Stimulation of the proliferation of mammalian

melanocytes is known to be elicited by PKC,[24] MAP kinase,[28] and PKA.[7,37] It remains to be investigated by a future study whether FFC activates these signaling pathways even in the absence of the growth factors or cytokines mentioned above.

Acknowledgements The author thanks Dr. or Mr. M. Akatsuka, K. Akatsuka, H. Kunoh, T. Nishimura, and M. Iinomi of the Institute for Biological Process Research of Akatsuka Co., Mie, Japan for their valuable discussions and generous supply of Korean ginseng, Chinese wolfberry, and Siberian ginseng extracts included in Pairogen Gold.

REFERENCES

1) Sugi, J. and Yamashita, S. (1991) The method for obtaining ferrous ferric chloride. *Bull. Soc. Sea Water Sci. Jpn.*, **42**, 11 (In Japanese).
2) Hasegawa, S., Meguro, A., Shimizu, M., Nishimura, T. and Kunoh, H. (2006) The ceramic bead that is suitable for a carrier of plant-rooting accelerator, *Streptomyces* sp. MBR-52. *Actinomycetologica*, **20**, 23–29.
3) Hirobe, T. (2007) Ferrous ferric chloride stimulates the proliferation and differentiation of cultured keratinocytes and melanocytes in the epidermis of neonatal mouse skin. *J. Health Sci.*, **53**, 576–584.
4) Hirobe, T. (1994) Keratinocytes are involved in regulating the developmental changes in the proliferative activity of mouse epidermal melanoblasts in serum-free culture. *Dev. Biol.,* **161**, 59–69.
5) Hirobe, T. (1992) Basic fibroblast growth factor stimulates the sustained proliferation of mouse epidermal melanoblasts in a serum-free medium in the presence of dibutyryl cyclic AMP and keratinocytes. *Development*, **114**, 435–445.
6) Hirobe, T. (1992) Melanocyte stimulating hormone induces the differentiation of mouse epidermal melanocytes in serum-free culture. *J. Cell. Physiol*, **152**, 337–345.
7) Hirobe, T. and Abe, H. (1999) Genetic and epigenetic control of the proliferation and differentiation of mouse epidermal melanocytes in culture. *Pigment Cell Res.*, **12**, 147–163.
8) Brain, J. (2006) Healthy aging: environmental factors, the role of drinking water and iron. *Abst. Antiaging Intl. Symp. Exposition Tokyo*, 14.
9) Hirobe, T. (1984) Histochemical survey of the distribution of the epidermal melanoblasts and melanocytes in the mouse during fetal and postnatal periods. *Anat. Rec.*, **208**, 589–594.
10) Mishima, Y. (1960) New technique for comprehensive demonstration of melanin, premelanin, and tyrosinase sites-combined dopa-premelanin reaction. *J. Invest. Dermatol.*, **34**, 355–360.
11) Hirobe, T. (1982) Genes involved in regulating the melanocyte and melanoblast-melanocyte populations in the epidermis of newborn mouse skin. *J. Exp. Zool.*, **223**, 257–264.
12) Mishima, Y. (1964) Electron microscopic cytochemistry of melanosomes and mitochondria. *J. Histochem. Cytochem.*, **12**, 784–790.
13) Hirobe, T. (1982) Origin of melanosome structures and cytochemical localizations of tyrosinase activity in differentiating epidermal melanocytes of newborn mouse skin. *J. Exp. Zool.*, **224**, 355–363.
14) Hirobe, T., Kawa, Y., Mizoguchi, M., Ito, S. and Wakamatsu, K. (2002) Effects of genic substitution at the pink-eyed dilution locus on the proliferation and differentiation of mouse epidermal melanocytes in vivo and in vitro. *J. Exp. Zool.*, **292**, 351–366.
15) Kim, K. M., Kwon, H. S., Jeon, S. G., Park, C. H., Sohn, S. W., Kim, D. I., Kim, S. S., Chang, Y. S., Kim, Y. K., Cho, S. H., Min, K. U. and Kim, Y. Y. (2008) Korean ginseng-induced occupational asthma and determination of IgE binding components. *J. Korean Med. Sci.*, **23**, 232–235.
16) Wu, B. Y., Zou, J. H. and Meng, S. C. (2003) Effect of wolfberry fruit and epimedium on DNA synthesis of the aging-youth 2B2 fusion cells. *Zhongguo Zhong Xi Yi Jie He Za Zhi*, **23**, 926–928.
17) Yeh, Y. C., Hahm, T. S., Sabliov, C. M. and Lo, Y. M. (2008) Effects of Chinese wolfberry (*Lycium chinense* P. Mill.) leaf hydrolysates on the growth of Pediococcus acidilactici. *Bioresour. Technol.*, **99**, 1383–1393.
18) Cicero, A. F., Derosa, G., Brillante, R., Bernardi, R., Nascetti, S. and Gaddi, A. (2004) Effects of Siberian ginseng (*Eleutherococcus senticosus* maxim.) on elderly quality of life: a randomized clinical trial. *Arch. Gerontol. Geriatr. Suppl.*, **9**, 69–73.
19) Jung, C. H., Jung, H., Shin, Y. C., Park, J. H., Jun, C. Y., Kim, H. M., Yim, H. S., Shin, M. G., Bae, H. S., Kim, S. H. and Ko, S. G. *Eleutherococcus senticosus* extract attenuates LPS-induced iNOS expression through the inhibition of Akt and JNK pathways in murine macrophage. *J. Ethnopharmacol.*, **113**, 183–187.
20) Niu, H. S., Hsu, F. L., Liu, I. M. and Cheng, J. T. (2007) Increase of β-endorphin secretion by syringin, an active principle of *Eleutherococcus senticosus*, to produce antihyperglycemic action in type 1-like diabetic rats. *Horm. Metab. Res.*, **39**, 894–898.

21) Niu, H. S., Liu, I. M., Cheng, J. T., Lin, C. L. and Hsu, F. L. (2008) Hypoglycemic effect of syringin from Eleutherococcus senticosus in streptozotocin-induced diabetic rats. *Planta Med.*, **74**, 109–113.
22) Liu, K. Y., Wu, Y. C., Liu, I. M., Yu, W. C. and Cheng, J. T. (2008) Release of acetylcholine by syringin, an active principle of *Eleutherococcus senticosus*, to raise insulin secretion in Wistar rats. *Neurosci. Lett.*, **434**, 195–199.
23) Maruyama, T., Kamakura, H., Miyai, M., Komatsu, K., Kawasaki, T., Fujita, M., Shimada, H., Yamamoto, Y., Shibata, T. and Goda, Y. (2008) Authentication of the traditional medicinal plant *Eleutherococcus senticosus* by DNA and chemical analyses. *Planta Med.*, **74**, 787–789.
24) Imokawa, G. (2004) Autocrine and paracrine regulation of melanocytes in human skin and in pigmentary disorders. *Pigment Cell Res.*, **17**, 96–110.
25) Imokawa, G., Kobayashi, T., Miyagishi, M., Higashi, K. and Yada, Y. (1977) The role of endothelin-1 in epidermal hyperpigmentation and signaling mechanisms of mitogenesis and melanogenesis. *Pigment Cell Res.*, **10**, 218–228.
26) Tada, A., Suzuki, I., Im, S., Davis, M. B., Cornelius, J., Babcock, G., Nordlund, J. J. and Abdel-Malek, Z. A. (1998) Endothelin-1 is a paracrine growth factor that modulates melanogenesis of human melanocytes and participates in their responses to ultraviolet radiation. *Cell Growth Differ.*, **9**, 575–584.
27) Hirobe, T. (2001) Endothelins are involved in regulating the proliferation and differentiation of mouse epidermal melanocytes in serum-free primary culture. *J. Invest. Dermatol. Symp. Proc.*, **6**, 25–31.
28) Coughlin, S. R., Barr, P. J., Cousens, L. S., Fretto, L. J. and Williams, L. T. (1988) Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity *in vivo*. *J. Biol. Chem.*, **263**, 988–993.
29) Halaban, R., Langdon, R., Birchall, N., Cuono, C., Baird, A., Scott, G., Moellmann, G. and McGuire, J. (1988) Basic fibroblast growth factor from human keratinocytes is a natural mitogen for melanocytes. *J. Cell Biol.*, **107**, 1611–1619.
30) Kunisada, T., Yoshida, H., Yamazaki, H., Miyamoto, A., Hemmi, H., Nishimura, E., Shultz, L. D., Nishikawa, S. I. and Hayashi, S. I. (1998) Transgene expression of steel factor in the basal layer of epidermis promotes survival, proliferation, differentiation and migration of melanocyte precursors. *Development*, **125**, 2915–2923.
31) Hirobe, T., Osawa, M. and Nishikawa, S. I. (2003) Steel factor controls the proliferation and differentiation of neonatal mouse epidermal melanocytes in culture. *Pigment Cell Res.*, **16**, 644–655.
32) Hirobe, T. (2002) Role of leukemia inhibitory factor in the regulation of the proliferation and differentiation of neonatal mouse epidermal melanocytes in culture. *J. Cell. Physiol.*, **192**, 315–326.
33) Imokawa, G., Yada, Y., Kimura, M. and Morisaki, N. (1996) Granulocyte/macrophage colony-stimulating factor is an intrinsic keratinocyte-derived growth factor for human melanocytes in UVA-induced melanosis. *Biochem. J.*, **313**, 625–631.
34) Hirobe, T., Furuya, R., Ifuku, O., Osawa, M. and Nishikawa, S. I. (2004) Granulocyte-macrophage colony-stimulating factor is a keratinocyte-derived factor involved in regulating the proliferation and differentiation of neonatal mouse epidermal melanocytes in culture. *Exp. Cell Res.*, **297**, 593–606.
35) Kunisada, T., Yamazaki, H., Hirobe, T., Kamei, S., Omoteno, M., Tagaya, H., Hemmi, H., Koshimizu, U., Nakamura, T. and Hayashi, S. I. (2000) Keratinocyte expression of transgenic hepatocyte growth factor affects melanocyte development, leading to dermal melanocytosis. *Mech. Dev.*, **94**, 67–78.
36) Hirobe, T., Osawa, M. and Nishikawa, S. I. (2004) Hepatocyte growth factor controls the proliferation of cultured epidermal melanoblasts and melanocytes from newborn mice. *Pigment Cell Res.*, **17**, 51–61.
37) Hirobe, T. (2005) Role of keratinocyte-derived factors involved in regulating the proliferation and differentiation of mammalian epidermal melanocytes. *Pigment Cell Res.*, **18**, 2–12.
38) Abdel-Malek, Z., Swope, V. B., Suzuki, I., Akcali, C., Harriger, M. D., Boyce, S. T., Urabe, K. and Hearing, V. J. (1995) Mitogenic and melanogenic stimulation of normal human melanocytes by melanotropic peptides. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 1789–1793.