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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

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Summary

Basic fibroblast growth factor (bFGF) stimulated the sustained proliferation of mouse epidermal melanoblasts derived from epidermal cell suspensions in a serum-free medium supplemented with dibutyryl adenosine 3′,5′-cyclic monophosphate (DBcAMP). The melanoblasts could be subcultured in the serum-free medium supplemented with the two factors in the presence of keratinocytes, but not in the absence of keratinocytes. In these conditions, some melanoblasts proliferated without differentiating for more than 20 days including a subculture. This is the first report of a successful culture of melanoblasts from mammalian skin. This culture system is expected to clarify further markers for melanoblasts and requirements for their proliferation and differentiation.

Key words: melanoblast, melanocyte, serum-free culture, basic fibroblast growth factor, adenosine 3′,5′-cyclic monophosphate, keratinocyte.

Introduction

In mice, melanoblasts, precursors of melanocytes, originate from the neural crest and migrate into the epidermis of all body regions in early embryonic life (Rawles, 1947). By 13 or 14 days of gestation, melanoblast colonisation of the epidermis is complete (Mayer, 1973). Mouse epidermal melanoblasts begin the production of unmelanised melanosomes at 14 days and begin to differentiate into melanocytes with the appearance of tyrosinase activity at 16 days of gestation (Hirobe, 1984). Melanocytes increase in number until 3 or 4 days after birth, and then their numbers decrease (Quevedo et al., 1966; Takeuchi, 1968; Weiss and Zelickson, 1975; Hirobe and Takeuchi, 1977, 1978; Hirobe, 1982a, 1984). However, little is known as to how the proliferation of epidermal melanoblasts is regulated during differentiation. Melanoblast cultures, serially passaged, provide sufficient cell numbers for such an analysis.

Several investigators have recently reported methods for culturing melanocytes from mammals including human (Eisinger and Marko, 1982; Wilkins et al., 1982; Gilchrest et al., 1984; Halaban and Alfano, 1984; Herlyn et al., 1987; Hirobe et al., 1988) and mouse (Sato et al., 1985; Abe et al., 1986; Bennett et al., 1987, 1989; Tamura et al., 1987; Halaban et al., 1988a; Hirobe, 1991). In these studies, enriched melanocyte cultures were obtained by culturing cells with 12-O-tetradecanoyl-13-acetate (TPA), bovine hypothalamic extract (BHE) or bovine pituitary extract (BPE). However, without exception, serum has been used to culture melanocytes. Serum contains numerous unknown factors in addition to mitogenic factors and nutrients. To overcome this problem, a serum-free culture system for melanocytes has been developed by several investigators (Halaban et al., 1987; Herlyn et al., 1988; Pittelkow and Shipley, 1989). They cultured human epidermal melanocytes in a serum-free medium supplemented with bFGF and DBcAMP, TPA and bFGF or BPE and TPA, respectively. However, there was no such system for culturing undifferentiated melanoblasts, which prompted me to develop a culture system to maintain and proliferate mouse epidermal melanoblasts in serum-free medium. Such a culture system may enable us to clarify the role of natural mitogenic factors in regulating the proliferation of melanoblasts during differentiation.

Materials and methods

Mice

House mice, *Mus musculus*, strain C57BL/10JHir, were given water, fed *ad libitum* on a commercial diet (Clea Japan, Tokyo, Japan) and maintained at 24±1°C with 40-60% relative humidity; 12 hours of fluorescent light were provided daily.

Primary culture of melanoblasts

The sources of tissues for melanoblast cultures were dorsal skins of 0.5-day-old mice. The skin was taken from the dorsolateral side of the trunk between the limbs. These tissues were cleaned of subcutaneous tissues and rinsed in calcium-, magnesium-free phosphate-buffered saline (CMF-PBS, pH 7.4). They were then cut into small pieces (5×5 mm^2) and incubated in a 0.25% trypsin (Gibco, Grand Island, NY, USA) solution in phosphate-buffered saline (PBS, pH 7.2) for 16-18 hours at 2°C. Epidermal sheets were mechanically separated from the dermis with fine forceps and floated onto a 0.02% ethylene-diamine-tetra-acetate (EDTA, Sigma, St. Louis, MO, USA) solution in CMF-PBS. The centrifuge tubes (Falcon, Lincoln Park, NJ, USA) were gently shaken to produce a basal cell suspension, and the cornified sheets were removed. They were then incubated at 37°C for 10 minutes. After this incubation, the epidermal cell suspensions were gently and repeatedly pipetted with Pasteur pipette to generate a single cell suspension. Undissociated cell clusters were removed by filtering them through steel mesh (Ikemoto, Tokyo, Japan). The EDTA solution containing a single cell suspension was diluted with CMF-PBS, and cells were pelleted by centrifugation (5 minutes at 1,500 revs minute^{-1}). The cell pellet was suspended in a Ham's F-10 (Gibco) medium and centrifuged at 1,500 revs minute^{-1} for 5 minutes. The cell pellet was resuspended in a melanoblast-proliferation medium (MPM) consisting of melanoblast-defined medium [MDM: F-10 plus 10 μg ml^{-1} of insulin (Ins, bovine, Sigma), 1 mg ml^{-1} of bovine serum albumin (BSA, Fraction V, Sigma), 1 μM ethanolamine (EA, Sigma), 1 μM phosphoethanolamine (PEA, Sigma), 50 μg ml^{-1} of gentamicin (Sigma) and 0.25 μg ml^{-1} of amphotericin B (Sigma)] supplemented with 0.5 mM dibutyryl adenosine 3′,5′-cyclic monophosphate (DBcAMP, Sigma, a membrane-permeable derivative of cAMP) and 2.5 ng ml^{-1} of basic fibroblast growth factor (bFGF, from bovine pituitary, Biomedical Technologies Inc., Stoughton, MA, USA). The cells in the epidermal cell suspension were counted in a haemocytometer chamber and plated onto plastic culture dishes (Lux, Naperville, IL, USA) at an initial density of 1×10^6 cells per 35 mm dish (1.11×10^5 cells cm^{-2}). Cultures were incubated at 37°C in a humidified atmosphere composed of 5% CO_2 and 95% air (pH 7.2). Medium was replaced by fresh medium four times a week. After 12-14 days, almost pure cultures of melanoblasts and melanocytes were obtained. In some cases, α-melanocyte-stimulating hormone (α-MSH, Sigma), 3-isobutyl-l-methylxanthine (IBMX, Sigma, a potent inhibitor of cAMP phosphodiesterase which catalyses cAMP to 5′-adenosine monosphosphate, Beavo et al., 1970), epidermal growth factor (EGF, from mouse submaxillary gland; Biomedical Technologies Inc.), acidic fibroblast growth factor (aFGF, from bovine brain, Biomedical Technologies Inc.), 7S nerve growth factor (NGF, from mouse submaxillary gland, Chemicon, Temecula, CA, USA), platelet-derived growth factor (PDGF, human, recombinant, Biomedical Technologies Inc.), transforming growth factor-α (TGF-α, Biomedical Technologies Inc.) and transforming growth factor-β_1 (TGF-β_1, King, Tokyo, Japan) were added to the culture medium to test their mitogenic activity toward melanocytes.

Primary culture of keratinocytes

Epidermal cell suspensions were obtained from 2.5-day-old mice by using the methods described above. The cell pellet after centrifugation was suspended in a Ca^{2+}-free Eagle's minimum essential medium (MEM, Gibco) and centrifuged at 1,500 revs minute^{-1} for 5 minutes. The cell pellet was resuspended in a keratinocyte-defined medium (KDM) consisting of Ca^{2+}-free MEM supplemented with MEM-non-essential amino acid solution (Gibco), 10 μg ml^{-1} of Ins, 1 mg ml^{-1} of BSA, 1 μM EA, 1 μM PEA, 1 μM hydrocortisone, 1 μM dexamethason and 0.03 mM $CaCl_2$ and the following antibiotics: 50 μg ml^{-1} of gentamicin; 0.25 μg ml^{-1} of amphotericin B. Initial density was 2×10^6 cells per 35 mm dish (2.22×10^5 cells cm^{-2}). After 3-4 days, almost pure (>95%) subconfluent (60-80% confluency) keratinocytes were obtained.

Secondary culture of melanoblasts

Primary cultures of melanoblasts and melanocytes were treated with a solution of 0.05% trypsin and 0.02% EDTA in CMF-PBS at 37°C for 15 minutes. After trypsinisation was inhibited by the addition of 2,000 U ml^{-1} of soybean trypsin inhibitor (Sigma), the cell suspensions were centrifuged at 1,500 revs minute^{-1} for 5 minutes. The cell pellet was resuspended in MPM or MPM supplemented with several growth factors at a density of 5×10^4 cells per 35 mm dish (5.56×10^3 cells cm^{-2}) and cultured.

Co-culture of melanoblasts and keratinocytes

Primary keratinocytes were similarly trypsinised and seeded into the secondary cultures of melanoblasts and melanocytes at a density of 2×10^5 cells per 35 mm dish (2.22×10^4 cells cm^{-2}) at 1 day, and cultured with MPM.

Melanoblast proliferation assay

The numbers of melanoblasts and melanocytes were determined per dish by using both phase-contrast and bright-field microscopy, and the calculations were based on the average number of cells from 10 randomly chosen microscopic fields covering an area of 0.581 mm^2. Bipolar, tripolar, dendritic, polygonal or epithelioid cells, as seen by phase contrast, which contained brown or black pigment granules, as observed by bright-field microscopy, were scored melanocytes. These cells were confirmed as melanocytes by dopa cytochemistry (Hirobe, 1982a). In contrast, bipolar, tripolar or dendritic cells, as seen by phase-contrast, which contained no pigments, as observed by bright-field microscopy, were scored melanoblasts. Almost all of these cells were stained by combined dopa-premelanin reaction (combined dopa-ammoniacal silver nitrate staining, Mishima, 1960; Hirobe, 1982a). The preferential staining reveals undifferentiated melanoblasts that contain stage I and II melanosomes in addition to tyrosinase-containing differentiated melanocytes (Mishima, 1964; Hirobe, 1982b).

Dopa and combined dopa-premelanin reactions

Mouse melanocyte cultures were fixed with 5% formalin in CMF-PBS at 2°C for 30 minutes, rinsed with distilled water, and incubated with 0.1% L-3, 4-dihydroxyphenylalanine (L-dopa, Wako, Osaka, Japan) solution in phosphate buffer (pH 6.8) at 37°C for 4 hours. They were then fixed with 10% formalin at 25°C for 1 hour, rinsed with distilled water and dried in air. For combined dopa-premelanin reaction, dried dishes after the dopa treatment were incubated with 10% ammoniacal silver nitrate (Wako) solution for 15 minutes at 58°C. After washing with distilled water, they were treated with 2% gold chloride (Wako) solution for 30 seconds at 25°C, and then transferred to 6% sodium thiosulfate (Wako) solution for 2 minutes at 25°C (Mishima, 1960). They were washed with distilled water and dried in air. Distilled water was added to the dish before microscopic observation or photography.

Results

Melanoblast proliferation in primary culture

Within 1 day after initiation of cultures with MPM, keratinocyte colonies could be seen in the dishes. Small bipolar, tripolar or dendritic cells were scattered between the keratinocyte colonies. A small number of cells possessed dark cytoplasm when examined by phase-contrast microscopy, and pigment granules were visible within them by using bright-field microscopy. Melanoblasts, which produced no pigments when examined under the bright-field microscope, were predominant. Melanoblasts and melanocytes were randomly distributed among the keratinocyte colonies. After 2 days, these presumed melanoblasts and melanocytes were in contact with the adjacent keratinocyte colony via a dendrite (Fig. 1A). After 3 days, the keratinocyte colonies increased in size and number, and melanoblasts increased in number (Fig. 2). From 3 days, melanoblasts engaged in mitotic division were frequently observed in the dishes (Fig. 1B,C). The mitotic indices of the melanoblasts are shown in Fig. 3. From 4-5 days, melanoblasts dramatically increased in number in the areas around the keratinocyte colonies (Fig. 1B). After 8-9 days, the keratinocyte colonies were smaller and refractile in appearance with progressive detachment of cells, whereas melanoblasts were more numerous than before (Figs 1C, 2). By 12-14 days, cultures were confluent and contained only melanoblasts or melanocytes (Fig. 1D). Pigment-producing melanocytes, which are dendritic, polygonal or epithelioid in morphology, were observed in the center of the melanoblast colony (Fig. 1D). On the contrary, undifferentiated melanoblasts, which are bipolar or tripolar in morphology, were observed around the melanocytes or at the periphery of the colony (Fig. 1D). The purity of the cultures of melanoblasts and melanocytes was greater than 99%. Melanoblasts and melanocytes gradually decreased in number after 14 days.

Dopa and combined dopa-premelanin reactions

Numerous cells positive to dopa (Fig. 4A,B) and to combined dopa-premelanin (Fig. 4C-F) reactions were

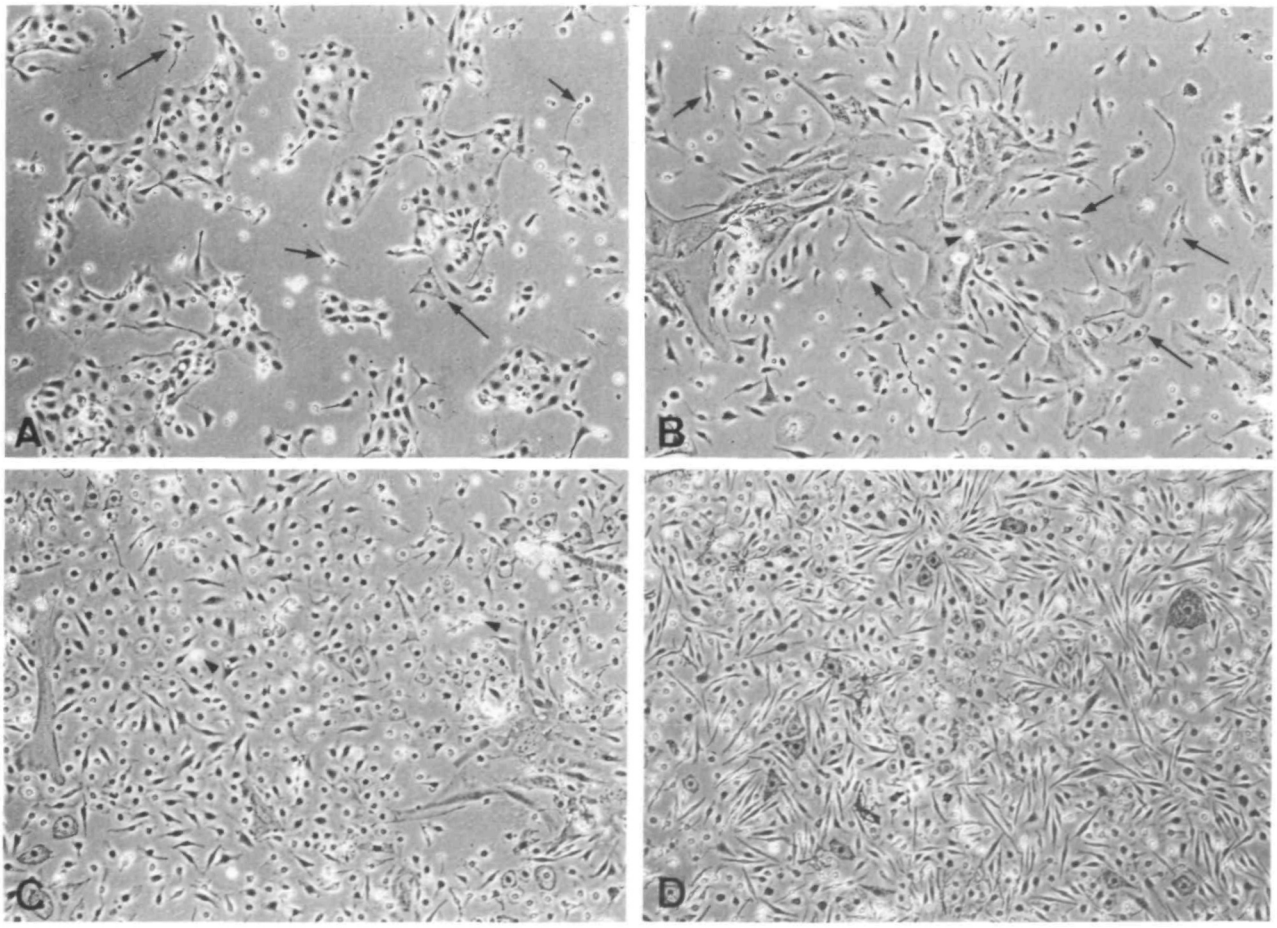

Fig. 1. Primary cultures of epidermal cell suspensions derived from mouse skin in MPM. (A) After 2 days in culture. Keratinocyte colonies and a smaller number of melanoblasts (short arrows) and melanocytes (long arrows) are evident. The melanoblasts are bipolar or tripolar. (B) After 6 days in culture. Melanoblasts (short arrows) have increased in number, and a small number of melanocytes (long arrows) are seen. Keratinocyte colonies increased in size and number. Mitotic melanoblasts (arrowhead) were often observed. (C) After 9 days in cultures. Numerous melanoblasts are seen. In contrast, keratinocyte colonies are shrinking. Arrowheads indicate mitotic melanoblasts. (D) After 12 days in culture. Enriched culture of pure melanoblasts and melanocytes. Phase-contrast microscopy. ×100.

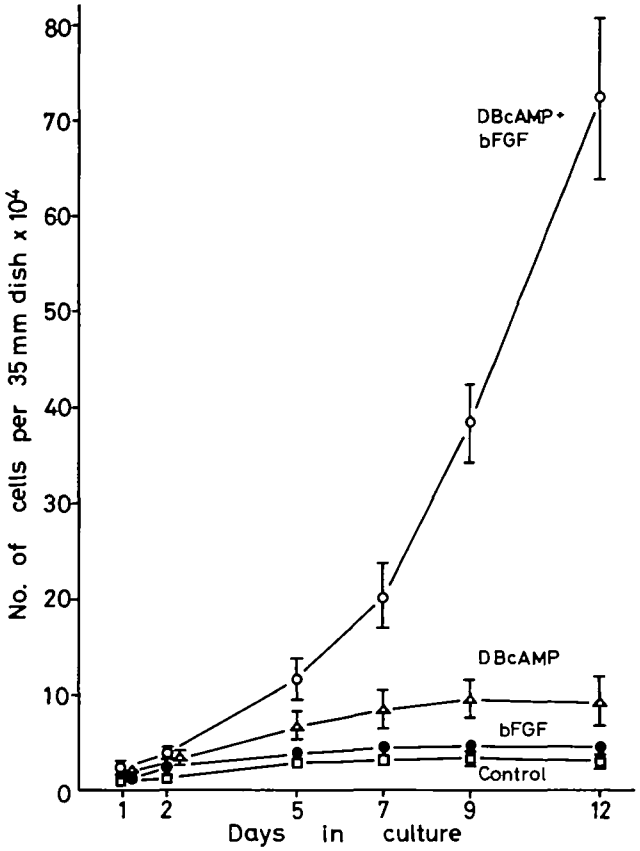

Fig. 2. Proliferation kinetics of mouse epidermal melanoblasts in primary culture. Epidermal cell suspensions were cultured in four different media: Control (MDM alone, □); 2.5 ng ml^{-1} of bFGF (●): 0.5 mM DBcAMP (△) and 2.5 ng ml^{-1} of bFGF plus 0.5 mM DBcAMP (○). The number of melanoblasts and melanocytes was counted at 1, 2, 5, 7, 9 and 12 days after plating. The epidermal cell suspensions of the four different groups were derived from the same litter of mice. The data are the averages of results from triplicate experiments. Each experiment was performed with different litters of mice. Bars indicate standard errors of the mean.

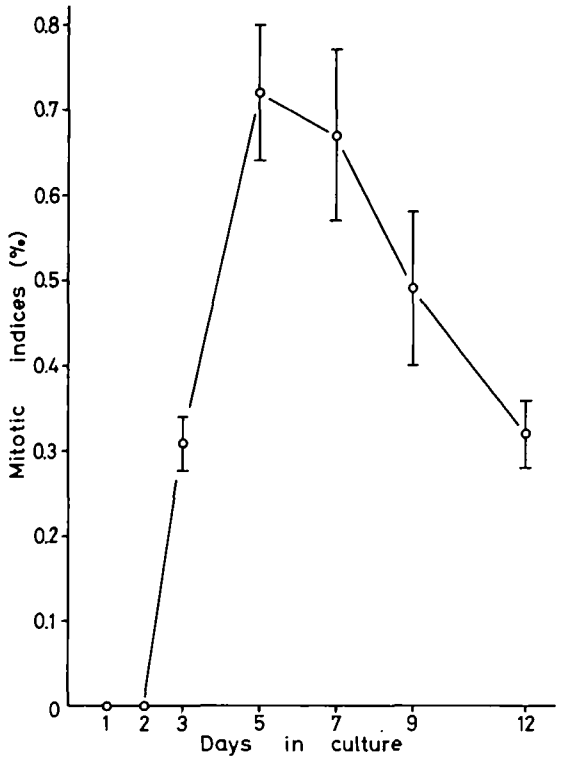

Fig. 3. Mitotic indices of epidermal melanoblasts cultured in MPM. Melanoblasts with mitotic figures were counted directly on the dishes by phase-contrast and bright-field microscopy. Maximal mitotic indices are observed at 5 days. The data are the averages of results from triplicate experiments. Each experiment was performed with different litters of mice. Bars indicate standard errors of the mean.

observed in the dishes cultured with MPM. The number of dopa-positive cells was comparable to that of pigment-producing melanocytes, suggesting that tyrosinase activity and pigments appear almost at the same time in the cultured melanoblasts. On the other hand, the number of cells positive to the combined dopa-premelanin reaction was comparable to that of melanoblasts plus melanocytes which were observed under the phase-contrast and bright-field microscopes, suggesting that almost all cells begin the production of stage I and II melanosomes by culturing with DBcAMP and bFGF.

Melanoblast proliferation kinetics in primary culture

Mouse melanoblasts and melanocytes cultured with MPM showed a proliferation phase from 2 to 12 days (Fig. 2). The number of melanoblasts and melanocytes observed at 12 days represented a 31-fold increase over the number of melanoblasts and melanocytes at 1 day. The proportion of pigment-producing melanocytes in the melanoblast-melanocyte population was about 20-30% at 12 days. The epidermal cell suspensions were also cultured in a medium that consisted of MDM supplemented with 0.5 mM DBcAMP (Fig. 2). Pure cultures of pigment-producing melanocytes were obtained with this medium (Fig. 5C). In this case, almost all cells obtained were differentiated melanocytes, but melanoblasts were rarely observed (Fig. 5C). In addition, the number of melanocytes observed at 12 days was about one-eighth to one-seventh (Fig. 2) as large as that of melanoblasts and melanocytes obtained with MPM. Melanoblasts slightly increased in number when the epidermal cell suspensions were cultured with MDM alone (Figs 2, 5A) or MDM supplemented with 2.5 ng ml^{-1} of bFGF (Figs 2, 5B). In these cases, almost all cells obtained were unpigmented melanoblasts, and only a few melanocytes (3-4%) were observed. These results show that the differentiation of mouse epidermal melanoblasts in culture can be stimulated by DBcAMP and can be reduced by bFGF, and that bFGF can stimulate the proliferation of melanoblasts in the presence of DBcAMP.

Effects of various doses of bFGF and DBcAMP

Epidermal cell suspensions were cultured with media that consisted of MDM supplemented with 0.5 mM

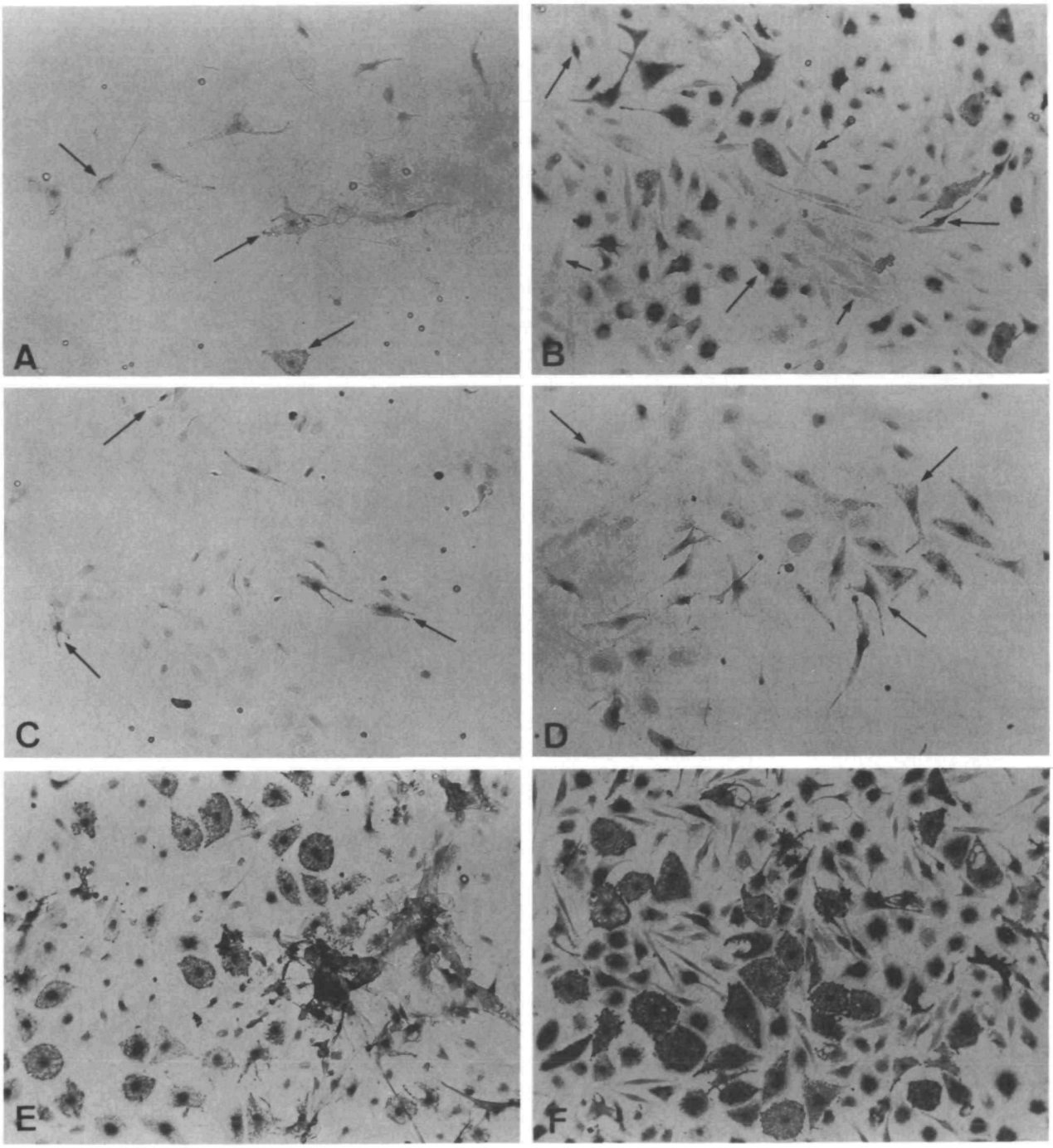

Fig. 4. Primary cultures of epidermal cell suspensions derived from mouse skin in MPM. Cultures were fixed at 2 (C), 5 (A, D), 9 (E) and 12 (B, F) days and incubated with dopa solution (A, B) or dopa-ammoniacal silver nitrate solution (combined dopa-premelanin reaction; C-F). Cells positive to the dopa reaction (long arrows; A, B) as well as cells positive to the combined dopa-premelanin reaction (long arrows; C-F) are shown. Cells negative to the dopa reaction (short arrows, B) are also shown. Bright-field microscopy. ×200.

DBcAMP plus bFGF at a dose of 0, 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5 or 10 ng ml^{-1}. The numbers of melanoblasts and melanocytes at 12 days with these concentrations were significantly ($P<0.05$) higher than in the control. Maximal number of melanoblasts and melanocytes was observed at the dose of 2.5 ng ml^{-1} (Fig. 6). The percentages of pigment-producing melanocytes in the melanoblast-melanocyte population from the dishes cultured with 0.5 mM DBcAMP plus 0.5 ng ml^{-1} of bFGF (Fig. 5D), 0.5 mM DBcAMP plus 2.5 ng ml^{-1} of bFGF (Fig. 5E) and 0.5 mM DBcAMP plus 10 ng ml^{-1} of bFGF (Fig. 5F) were 49.75±5.33, 25.35±3.33,

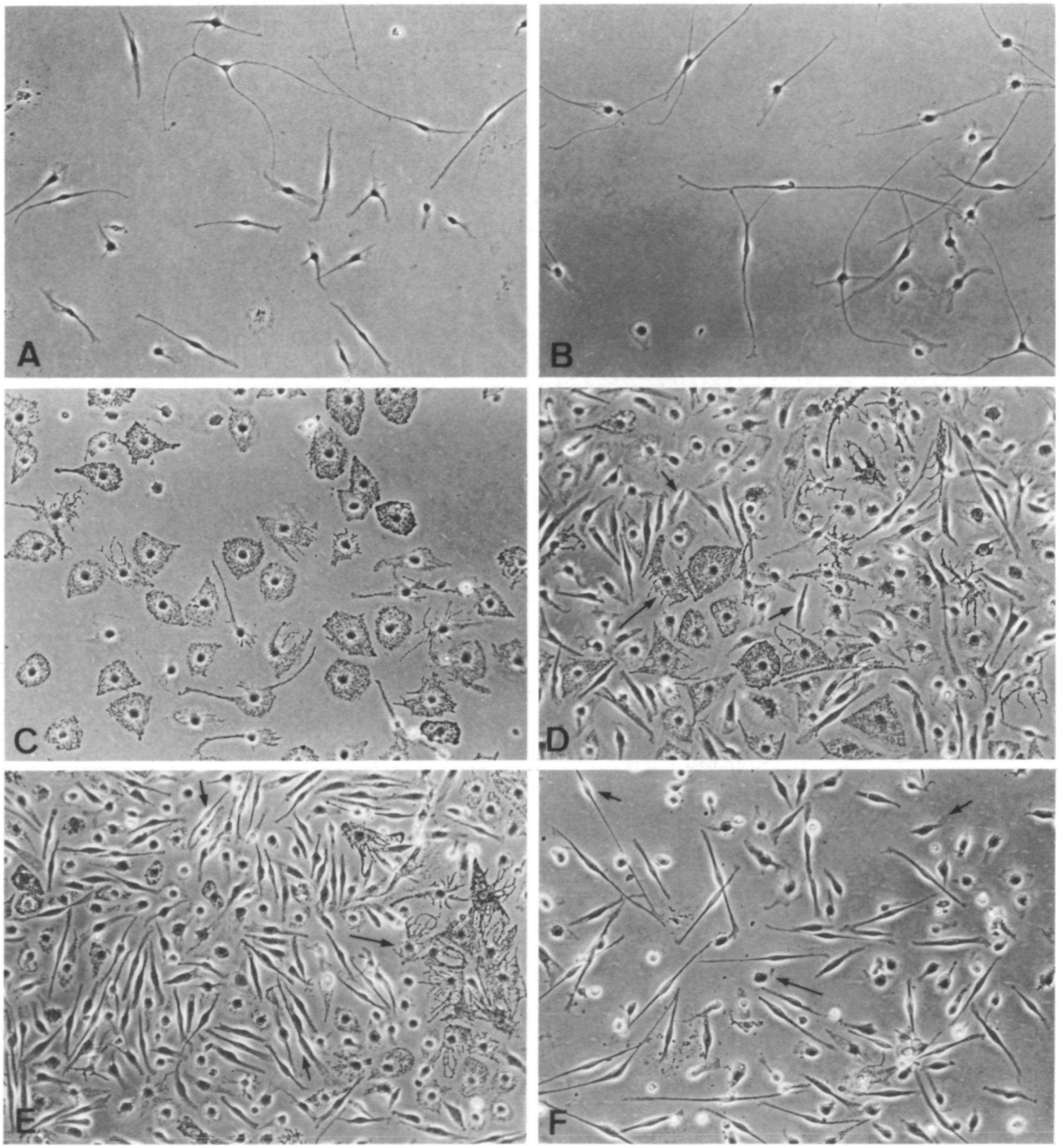

Fig. 5. Primary cultures of epidermal cell suspensions derived from mouse skin in six different media: Control (MDM alone, A), 2.5 ng ml^{-1} of bFGF (B), 0.5 mM DBcAMP (C), 0.5 ng ml^{-1} of bFGF plus 0.5 mM DBcAMP (D), 2.5 ng ml^{-1} of bFGF plus 0.5 mM DBcAMP (E) and 10 ng ml^{-1} of bFGF plus 0.5 mM DBcAMP (F). After 12 days, pure melanoblast cultures were obtained in the dishes cultured with MDM (A) or 2.5 ng ml^{-1} of bFGF (B). In contrast, pure melanocyte cultures were obtained in the dishes cultured with 0.5 mM DBcAMP (C). Pure cultures of melanoblasts (short arrows) and melanocytes (long arrows) were obtained in the dishes cultured with bFGF and DBcAMP (D-F). However, the percentage of differentiated melanocytes in the melanoblast-melanocyte population decreased with increasing concentrations of bFGF. Phase-contrast microscopy. ×200.

10.74±2.26% (Mean±standard error of the mean, $n=3$), respectively. These results show that the differentiation of epidermal melanocytes can be inhibited with increasing concentrations of bFGF in the presence of DBcAMP.

Epidermal cell suspensions were similarly cultured

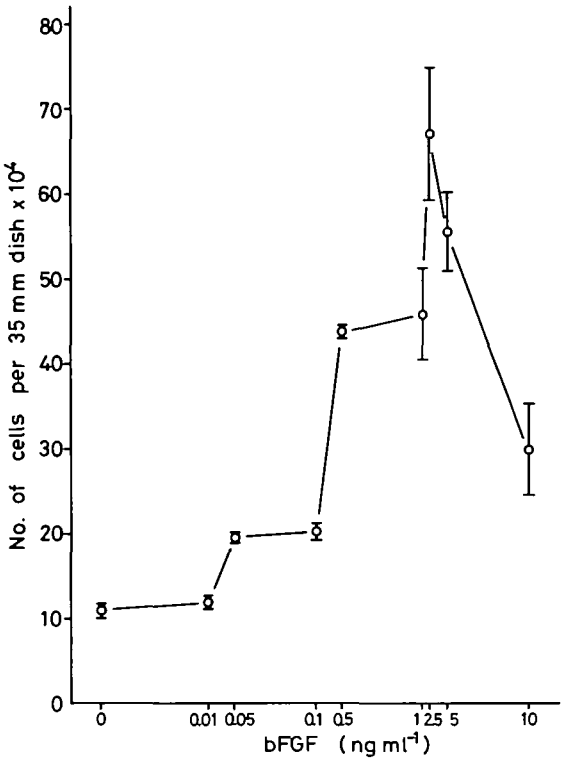

Fig. 6. Dose-response curve for the proliferation of mouse epidermal melanoblasts cultured for 12 days in a medium that consisted of MDM supplemented with 0.5 mM DBcAMP plus bFGF at various doses (0-10 ng ml^{-1}). The epidermal cell suspensions of the nine different groups were derived from the same litter of mice. The data are the averages of results from triplicate experiments. Each experiment was performed with different litters of mice. Bars indicate the standard errors of the mean.

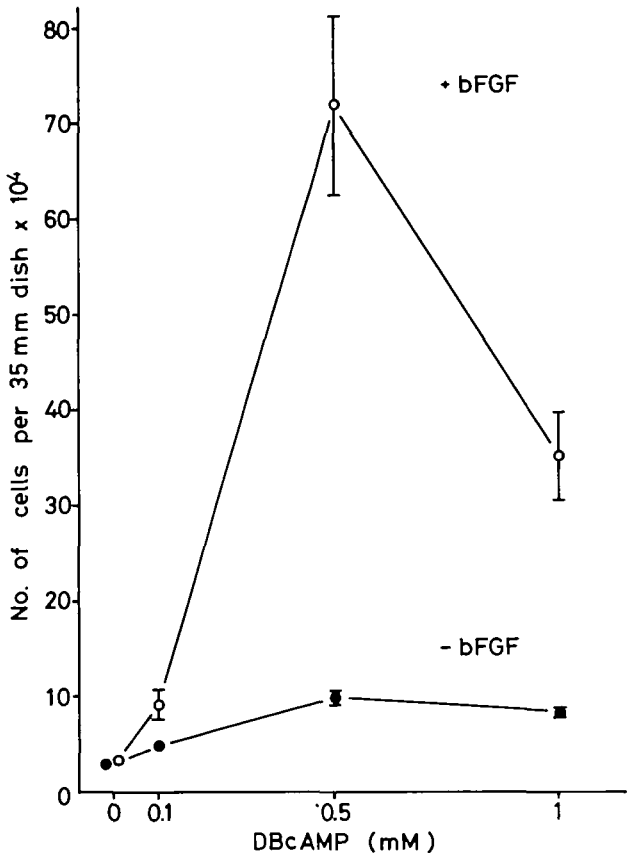

Fig. 7. Dose-response curve for the proliferation of mouse epidermal melanoblasts cultured for 12 days in a medium that consisted of MDM plus DBcAMP at various doses (0-1 mM) with (○) or without (●) 2.5 ng ml^{-1} of bFGF. Maximal effects of DBcAMP on the proliferation of melanoblasts were observed at the dose of 0.5 mM either in the presence or in the absence of bFGF. The epidermal cell suspensions of the eight different groups were derived from the same litter of mice. The data are the averages of results from triplicate experiments. Each experiment was performed with different litters of mice. Bars indicate the standard errors of the mean.

with media that consisted of MDM supplemented with 2.5 ng ml^{-1} of bFGF plus DBcAMP at a dose of 0, 0.1, 0.5 and 1 mM. The numbers of melanoblasts and melanocytes at 12 days cultured in these concentrations were significantly ($P<0.05$) higher than in control. Maximal number of melanoblasts and melanocytes was observed at the dose of 0.5 mM (Fig. 7). Epidermal cell suspensions were also cultured with media that consisted of MDM plus DBcAMP at a dose of 0, 0.1, 0.5 and 1 mM. Almost all cells obtained were differentiated melanocytes and the numbers of melanocytes at 12 days cultured in these concentrations of DBcAMP were significantly ($P<0.05$) higher than those of melanoblasts and melanocytes cultured in MDM. Maximal number of melanocytes was observed at the dose of 0.5 mM (Fig. 7).

Effects of DBcAMP, α-MSH and IBMX

Epidermal cell suspensions were cultured with media that consisted of MDM supplemented with 2.5 ng ml^{-1} of bFGF plus DBcAMP, α-MSH or IBMX. The numbers of melanoblasts and melanocytes at 12 days cultured in DBcAMP (0.1, 0.5 mM) were significantly ($P<0.05$) higher than those in control (Table 1). Almost all cells obtained were differentiated melanocytes in the dishes cultured with α-MSH, α-MSH plus IBMX or IBMX and the number of melanocytes did not differ significantly from that in control (Table 1). These results show that bFGF can stimulate the proliferation of melanoblasts at high cAMP levels. MSH alone, IBMX alone or combined treatment of MSH and IBMX is thought to be unable to maintain the concentration of cAMP high enough to induce the proliferation of melanoblasts.

Effects of various growth factors

aFGF (0.1, 1, 10 and 25 ng ml^{-1}), EGF (1, 10 and 100 ng ml^{-1}), NGF (1, 10 and 100 ng ml^{-1}) and PDGF (1, 10 and 100 ng ml^{-1}) were tested for their mitogenic activity toward melanoblasts. All of these factors failed to stimulate the proliferation of melanoblasts (Table 2). Almost all cells obtained were differentiated melanocytes. Similarly, the combination of MPM with these

Table 1. *Effects of DBcAMP, α-MSH and IBMX on the proliferation of mouse epidermal melanoblasts in primary culture*

Groups	No. of cells ($\times 10^4$)
Control	4.28±0.72[a]
DBcAMP (0.1 mM)	9.54±1.34[b]
DBcAMP (0.5 mM)	78.10±10.75[c]
α-MSH	4.32±0.52[d]
α-MSH + IBMX	5.04±0.25[e]
IBMX	3.44±0.68[f]

Note: Epidermal cell suspensions were cultured with six different media: control (MDM plus 2.5 ng ml^{-1} of bFGF), DBcAMP (0.1, 0.5 mM), α-MSH (10 nM), α-MSH (10 nM) plus IBMX (0.1 mM) and IBMX (0.1 mM). The number of melanoblasts and melanocytes was counted at 12 days. The epidermal cell suspensions of the six different groups were derived from the same litter of mice. The data are the averages of results from triplicate experiments. Each experiment was performed with different litters of mice. Each value is the mean ± standard errors of the mean.

t-test: a-d, a-e, a-f, d-e, d-f, e-f; Nonsignificant differences. a-b, a-c, b-d, b-e, b-f, b-c, c-d, c-e, c-f; Significant differences ($P < 0.05$).

Table 2. *Effects of various growth factors on the proliferation of epidermal melanoblasts in primary culture*

Growth factors	Dose (ng ml^{-1})	No. of cells ($\times 10^4$)
Control	–	9.84±1.01
bFGF	2.5	65.29±7.53
aFGF	0.1	11.38±0.61
	1	10.29±0.20
	10	9.42±0.63
	25	7.73±0.26
EGF	1	7.31±0.98
	10	8.08±0.74
	100	8.59±0.33
NGF	1	6.49±0.38
	10	7.36±1.62
	100	6.24±0.38
PDGF	1	9.24±1.25
	10	9.96±1.49
	100	8.43±0.70

Note: Epidermal cell suspensions were cultured with fifteen different media: control (MDM plus 0.5 mM DBcAMP), bFGF (2.5 ng ml^{-1}), aFGF (0.1, 1, 10, 25 ng ml^{-1}), EGF (1, 10, 100 ng ml^{-1}), NGF (1, 10, 100 ng ml^{-1}) and PDGF (1, 10, 100 ng ml^{-1}). The number of melanoblasts and melanocytes was counted at 12 days. Significant ($P<0.05$) increase in the number of cells was observed only in the culture with bFGF. The epidermal cell suspensions of the fifteen different groups were derived from the same litter of mice. The data are the averages of results from triplicate experiments. Each experiment was performed with different litters of mice. Each value is the mean ± standard errors of the mean.

factors brought about no increase in the proliferation of melanoblasts (results not shown).

Co-culture of melanoblasts and keratinocytes

Within 1 day after initiation of culture with KDM, keratinocyte colonies could be seen, whereas melanoblasts and melanocytes could be hardly seen. After 2 days, the keratinocyte colonies increased in size and number, and subconfluent keratinocyte cultures (Fig. 8A) were obtained at 3-4 days. The purity of keratinocytes was over 95%. On the other hand, enriched cultures of pure melanoblasts and melanocytes were trypsinised and cultured with MPM (secondary culture). Subconfluent primary keratinocytes were trypsinised and seeded into the secondary cultures of melanoblasts and melanocytes at 1 day, and cultured with MPM (Fig. 8B). Melanoblasts increased in number with a similar time schedule to the primary culture (Fig. 9). Melanoblasts dramatically increased in number and mitotic melanoblasts were often observed in the areas around the keratinocyte colonies (Fig. 8C,D). In contrast, melanoblasts in secondary culture failed to proliferate in the absence of keratinocytes (Fig. 9). Moreover, conditioned medium (CM) prepared from the keratinocyte-enriched primary cultures (3-5 days) in MPM or from the subconfluent keratinocyte primary cultures in KDM failed to proliferate melanoblasts in secondary cultures (results not shown). When the secondary cultures of epidermal melanoblasts were subcultured, they proliferated in the presence of keratinocytes. However, the rate of proliferation of melanoblasts slowed.

aFGF (0.1, 1, 100 and 25 ng ml^{-1}), EGF (1, 10 and 100 ng/ml^{-1}), NGF (1, 10 and 100 ng ml^{-1}), PDGF (1, 10 and 100 ng ml^{-1}), TGF-α (0.001, 0.01, 0.1, 1 and 10 ng ml^{-1}) and TGF-β_1 (0.001, 0.01, 0.1, 1 and 10 ng ml^{-1}) were tested for their mitogenic activity towards melanocytes in secondary culture. None of these factors replaced the mitogenic effects of keratinocytes (results not shown).

Discussion

In the present study, bFGF stimulated the sustained proliferation of mouse epidermal melanoblasts in serum-free medium in the presence of both DBcAMP and keratinocytes. The melanoblasts were operationally defined as the unpigmented cells that react with the stain for immature melanosomes but not for tyrosinase. In these culture conditions, some melanoblasts proliferated without differentiating for at least 20 days including a subculture. This is the first report that the undifferentiated melanoblasts could be maintained and proliferated in serum-free culture. This culture system may be a useful tool for studying further markers for melanoblasts and requirements for their proliferation and differentiation.

Halaban et al. (1987) reported that bFGF was mitogenic to human epidermal melanocytes in the presence of DBcAMP. The reason why bFGF failed to stimulate the proliferation of mammalian melanoblasts or melanocytes in the absence of DBcAMP is not known at present. The dependence of bFGF-stimulated proliferation on the presence of DBcAMP may be unique to mammalian melanoblasts and Schwann cells (Davis and Stroobant, 1990). It has not been observed

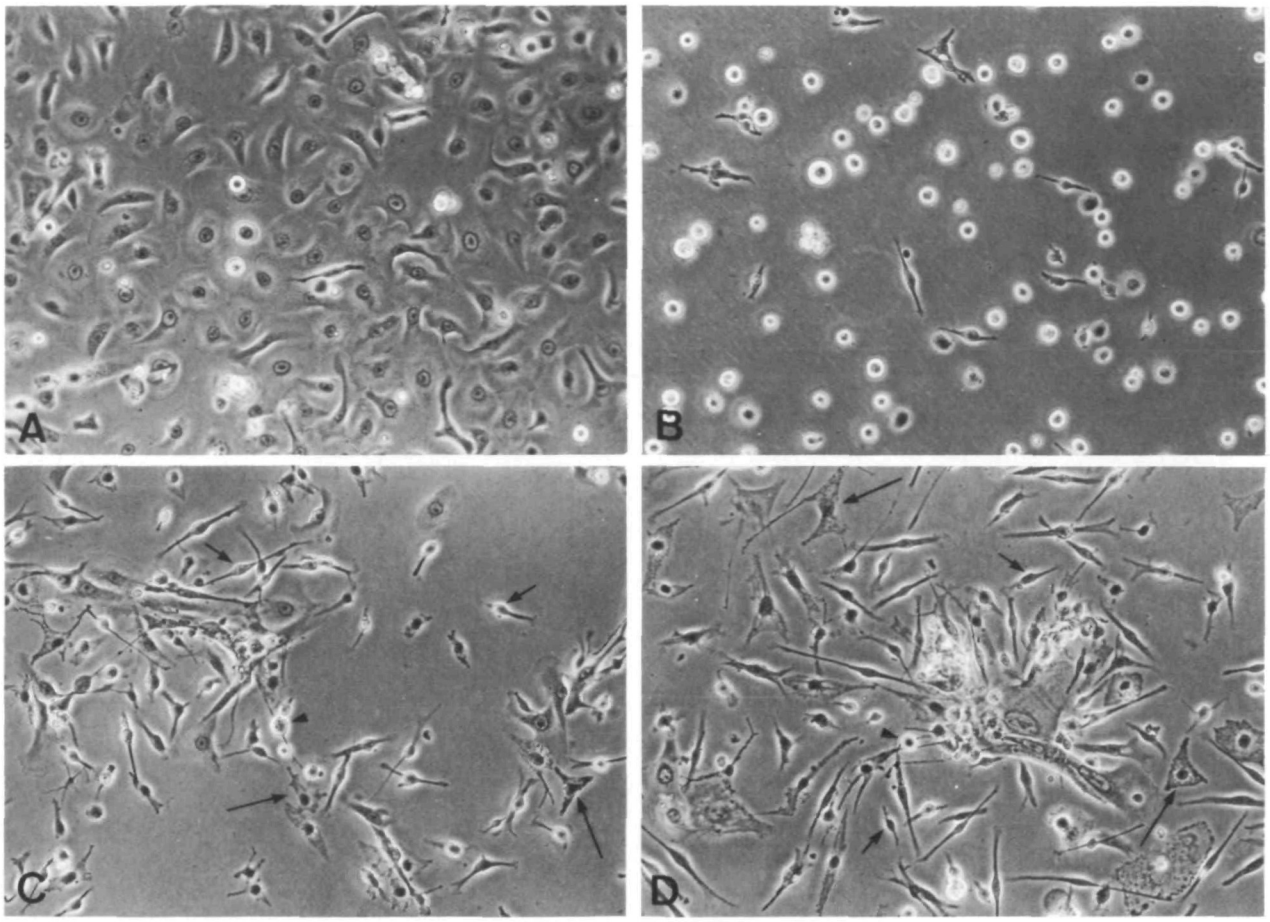

Fig. 8. Co-culture of melanocytes and keratinocytes. Epidermal cell suspensions were cultured with KDM. Pure and enriched cultures of keratinocytes were obtained after 3 days (A). Epidermal cell suspensions were also cultured with MPM. Pure cultures of melanoblasts and melanocytes were obtained after 12 days. Melanoblasts and melanocytes were trypsinised and cultured with MPM. The subconfluent primary keratinocytes (A) were trypsinised and seeded at a density of 2×10^5 cells per dish into the secondary cultures of pure melanoblasts and melanocytes at 1 day, and cultured with the MPM (B). (C) After 2 days in culture. Keratinocyte colonies and numerous melanoblasts (short arrows) are evident. A small number of melanocytes (long arrows) are also seen. Mitotic melanoblasts (arrowhead) were often observed. (D) After 6 days in culture. Melanoblasts (short arrows) have increased in number. Melanocytes (long arrows) are also seen. Mitotic melanoblasts (arrowhead) were often observed. Phase-contrast microscopy. ×200.

in other cells (review: Baird et al., 1986). Moreover, a bimodal proliferative response to both bFGF and DBcAMP was observed, i.e., high doses were less mitogenic than moderate ones. In addition, the proliferation-stimulating effect of DBcAMP was not replaced by α-MSH or IBMX in this study. One possible explanation is that neither MSH nor IBMX is able to maintain the concentrations of cAMP high enough to induce the proliferation of melanoblasts. The present study also demonstrated that the differentiation of mouse epidermal melanoblasts was reduced by bFGF and by omitting DBcAMP. The results indicate the possibility that the proliferation and differentiation of mouse epidermal melanoblasts are regulated by both bFGF and cAMP.

DeLuca et al. (1988) found that the human epidermal keratinocytes stimulated the proliferation of human epidermal melanocytes. Gordon et al. (1989) reported that CM derived from pure cultures of human keratinocytes enhanced the proliferation of human epidermal melanocytes in the presence of BHE. Hirobe (1991) also reported that CM prepared from keratinocyte-enriched cultures possessed an activity that stimulated the proliferation of mouse epidermal melanocytes in the presence of BPE. These results as well as the present results suggest that the keratinocytes produce factors that induce the proliferation of mammalian melanoblasts or melanocytes. In some culture systems (Halaban et al., 1988b), bFGF replaced the proliferation-stimulating effect of keratinocytes, but not in other culture systems (Gordon et al., 1989; Hirobe, 1991). In the present study, mouse epidermal melanoblasts proliferated in the areas around the keratinocyte colonies both in the primary and secondary cultures. Moreover, the conditioned medium prepared from the keratinocyte-enriched cultures failed to proliferate melanoblasts. These results suggest that the stimulation of melanoblast proliferation by keratinocytes requires a

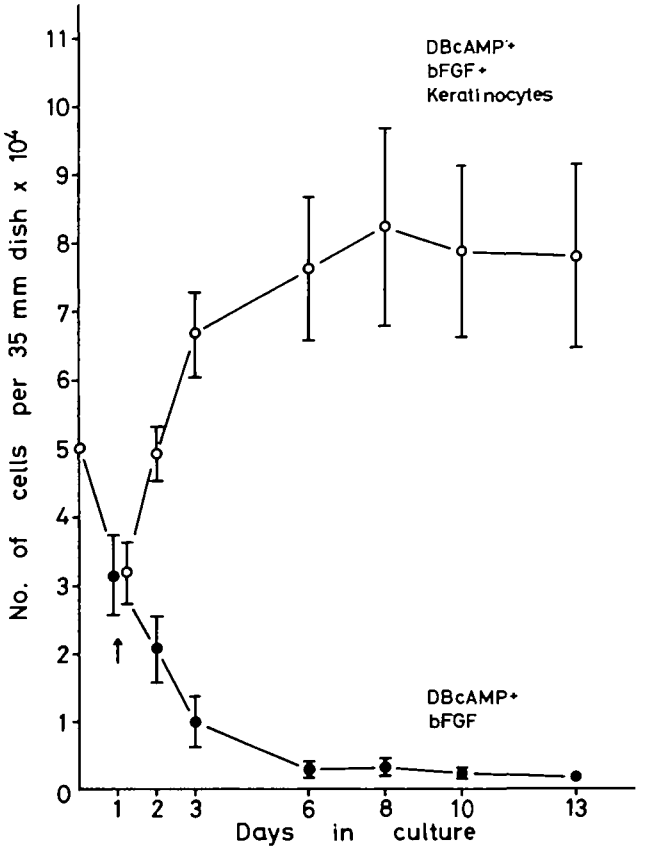

Fig. 9. Proliferation kinetics of mouse epidermal melanoblasts in secondary culture. Pure and enriched primary cultures of melanoblasts and melanocytes were trypsinised and cultured with MPM with (○) or without (●) keratinocytes. Primary keratinocytes were trypsinised and seeded into the secondary culture of melanoblasts and melanocytes at 1 day (arrow). The number of melanoblasts and melanocytes was counted at 1, 2, 5, 7, 9 and 12 days after co-culture with keratinocytes. The epidermal cell suspensions of the two different groups were derived from the same primary culture. The data are the averages of results from triplicate experiments. Each experiment was performed with different primary cultures. Bars indicate standard errors of the mean.

direct contact between melanoblasts and keratinocytes. Therefore, it is reasonable to assume that the mitogenic factor derived from keratinocytes is not a paracrine factor, but a membrane-bound factor. Recently, a new growth factor has been shown that is the ligand for the receptor encoded by the c-*kit* proto-oncogene (*W*). This stem cell factor (SCF)/Steel factor is known to be present in the skin (review: Witte, 1990). SCF is expressed during embryogenesis in the cells associated with both the migratory pathway and homing sites of melanoblasts (Matsui et al., 1990). Moreover, there is a membrane-bound form of SCF that may be required by melanoblasts as suggested by the depigmentation in Steel-Dickie mice (Flanagan et al., 1991). These results suggest the possibility that the unknown mitogen produced by keratinocytes is SCF, but this needs further investigation.

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