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A study of the effects of phorbol 12-myristate-13-acetate on cell differentiation of pure human melanocytes in vitro

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Summary. Pure human melanocyte cultures were established in a serum-free medium containing epidermal growth factor (10 ng/ml), hydrocortisone (10^{-7} *M*), insulin (5 µg/ml), transferrin (10 µg/ml), cholera toxin (2 ng/ml), isobutylmethyl xanthine (10^{-4} *M*) and bovine pituitary extract (30 µg/ml). To investigate the biological effects of PMA on melanocytes in vitro, the cells were incubated in media containing various concentration of PMA (including 0 n*M*, 85 n*M* and 170 n*M*), and grown continuously for 12 days without passage. The cells were observed for changes in cell morphology, cell cycle, cytoskeleton and HLA-DR expression. In addition, the effect of PMA on tyrosinase activity was also evaluated. The results revealed that the higher the PMA concentration, the higher the percentage of large irregularly shaped melanocytes. These large melanocytes were three to ten times the size of small bipolar or multipolar cells. A higher concentration of PMA was also associated with a higher percentage of melanocytes in the S and G2-M phases of the cell cycle and with a higher percentage of melanocytes as tetraploid and octaploid karyotypes. The cytoskeleton (vimentin) in the large irregularly shaped cells appeared disaggregated as compared with that in the usual dendritic cells with a compact distribution. HLA-DR was found to be expressed on some melanocytes growing in media containing PMA, appearing both in large dendritic cells and large irregularly shaped cells. None of the cells expressed HLA-DR when cultured in the abscence of PMA. PMA at 85 n*M*, but not at 170 n*M*, significantly stimulated tyrosinase activity as compared with the controls (0 n*M* PMA). It thus seems appropriate to study the carcinogenic or other properties of melanocytes in systems that do not contain PMA, such as the serum-free medium proposed by us.

Key words: Phorbol 12-myristate – 13-acetate – Melanocyte – Cell differentiation

Pure normal human melanocytes can be grown in culture in the presence of either a phorbol ester tumour promoter [2] or bovine pituitary extract (BPE) [4, 18], and substances that increase intracellular levels of cyclic adenosine monophosphate (cAMP), such as cholera toxin (CT) [2] or isobutylmethyl xanthine (IBMX), to enhance proliferation [5]. Recently, it has been proposed that basic fibroblast growth factor (bFGF) is the natural growth factor for human melanocytes [6].

Phorbol esters, such as phorbol 12-myristate-13-acetate (PMA), are potent tumour promoters, which elicit various biochemical and biological responses in mouse skin [15] and in cultured cells [1, 17]. Before the utilization of BPE, PMA is necessary for pure human melanocyte culture, but, as a potent tumor promoter, may elicit biological and biochemical response in the cultured melanocytes. Previously, it was reported that PMA resulted in a dose-related inhibition of growth and a stimulation of differentiated functions of cultured human melanoma [9]. However, little is reported about the biological effects of PMA on cultured human melanocytes. It is still an unresolved question whether the melanocytes grown in PMA are biologically normal and suitable for subsequent use in various experiments such as melanocyte transplantation [12]. In this study, we investigated the effects of PMA on cultured human melanocytes (including changes in cell morphology, growth curve, cycle, and markers), and evaluated the degree of cell alteration associated with various concentration of PMA.

Materials and methods

Cell culture

The tissue for the human melanocyte culture was obtained from adult foreskins from routine circumcision. The skin was cleaned of excess subcutaneous tissue, cut into small pieces (5 × 5 mm) and incubated overnight at 4 °C in calcium- and magnesium-free phosphate-buffered saline (PBS) (pH 7.2) containing 0.25% trypsin (Gibco, Santa Clara, Calif., USA). Epidermal sheets were mechanically separated from the dermis using fine forceps. An epidermal cell suspension was prepared using a previously described method

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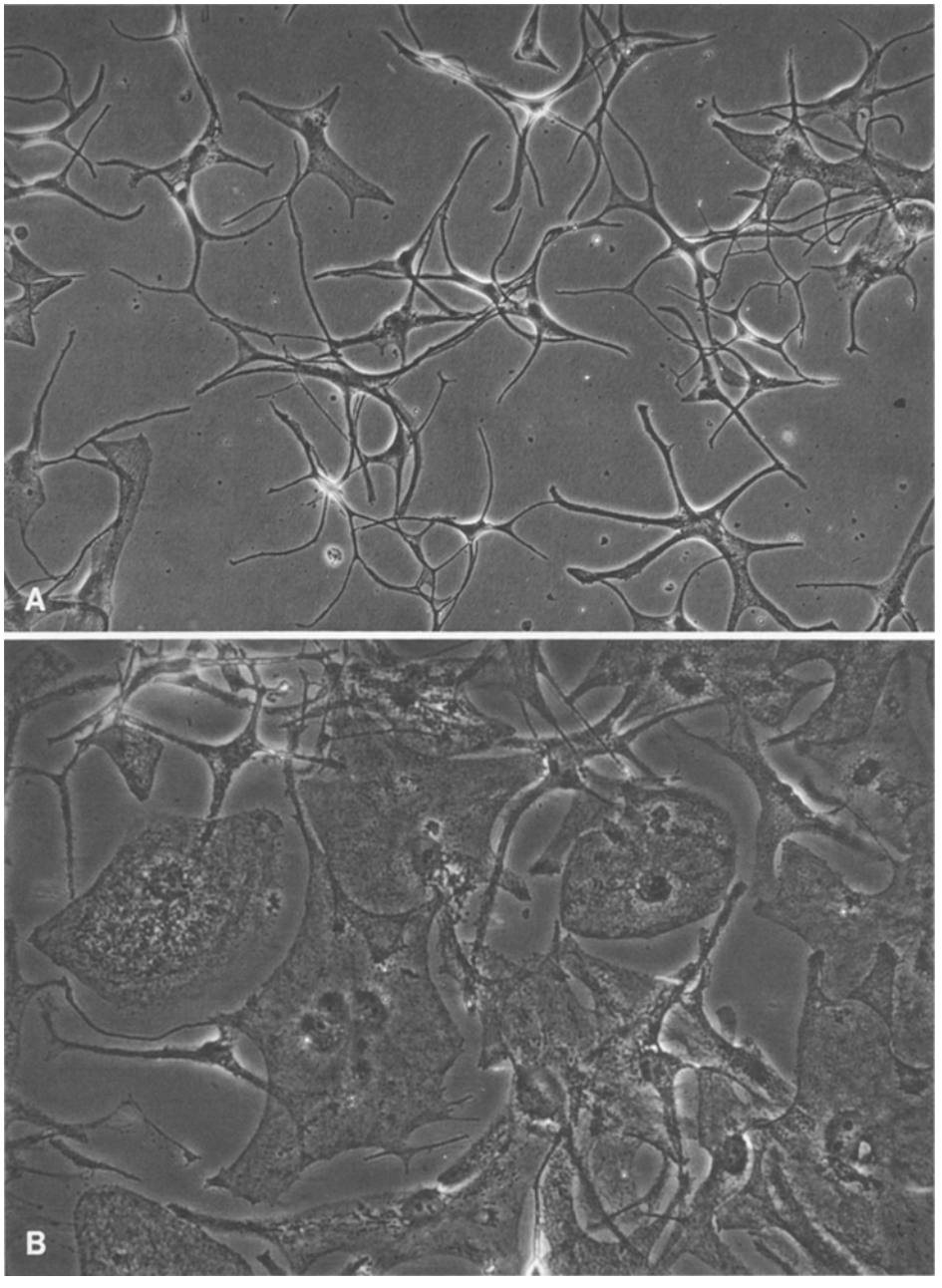

Fig. 1. The melanocytes appear as small bipolar or multipolar shapes when growing in SFM medium without PMA (**A**). In contrast, some of the cells growing in SFM containing 170 n*M* PMA appear as large irregular shapes, with one or multiple nuclei and prominent nucleoli (**B**). ×400

[2]. Cells were pelleted by centrifugation (500 *g*, 10 min) and resuspended in a serum-free medium (SFM) containing MCDB151 medium (Gibco) supplemented with growth factors and hormones including epidermal growth factor (EGF) 10 ng per ml, hydrocortisone (HC) 10^{-7} *M*, insulin (I) 5 µg per ml, CT 2 ng per ml, transferrin (T) 10 µg per ml, BPE 30 µg per ml, IBMX 10^{-4} *M*, penicillin 100 U per ml, streptomycin 100 µg per ml. The CT and IBMX were purchased from Sigma (St. Louis, Mo., USA) and the EGF, HC, I, T and BPE were purchased from Collaborative Research, Bedford, Mass. The epidermal cell suspension was counted in a haemocytometer chamber, plated onto plastic flasks (Falcon Plastics, Oxnard, Calif.) at a seeding density of $5-10 \times 10^5$ cells/ml, and incubated at 37 °C in a humidified atmosphere containing 8% CO_2. After 24 h, and then every 2 days, the medium was changed to remove unattached cells. After about 10 days a pure human melanocyte culture was obtained, as identified by dopa reaction and electron microscopic examination [8].

Serial subculture

The semiconfluent purified human melanocytes were treated with 0.25% trypsin solution in PBS at 37 °C for 20 min, harvested with fetal bovine serum (FBS) (Gibco), centrifuged at 500 *g* for 10 min, and resuspended in SFM. The resulting cells were counted in a haemocytometer chamber and reinoculated at a density of 5.5×10^4 cell/ml. After 24 h the medium was changed. Cultures were repassaged every 5–7 days. After the fourth passage the melanocyte culture was used to evaluate the effects of PMA on melanocytes in vitro.

Experimental procedure

The fourth-passaged melanocytes in cell suspension were counted in a haemocytometer chamber, 3.42×10^4 melanocytes were added

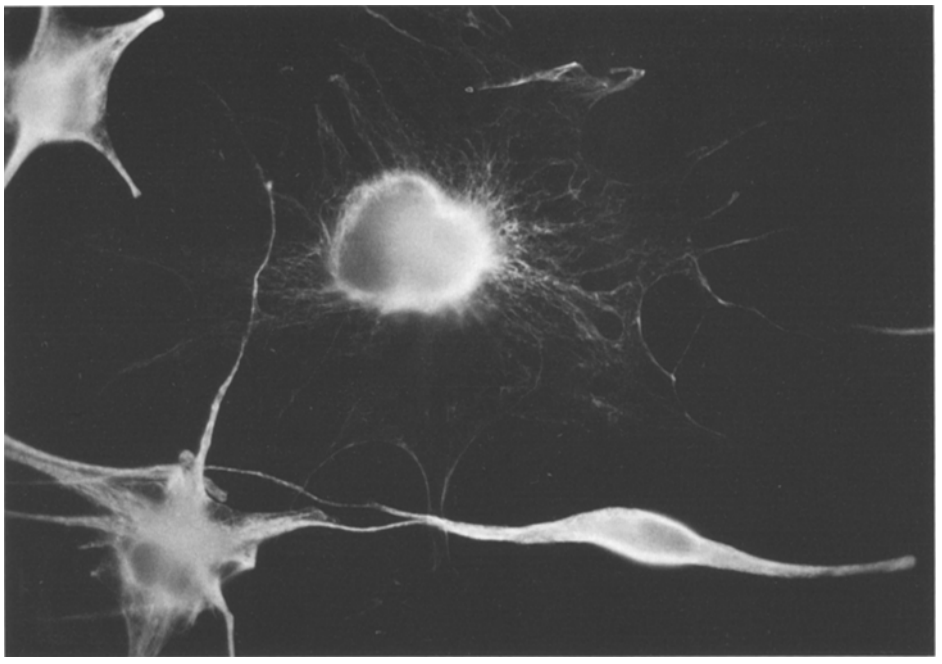

Fig. 2. The cytoskeleton (vimetin) of melanocytes. In large irregularly shaped cells the vimentin is disaggregated and in usual dendritic cells the vimetin is compact. Direct immunofluorescence, ×400

Table 1. The analysis of cell cycle of melanocytes cultured in triplicate in SFM without PMA, and subcultured every 5 to 7 days. The cells from the first, second and third passaged cultures were collected and analysed

	First passage (%)	Second passage (%)	Third passage (%)
G0-G1	70.3 ± 0.5	67.2 ± 0.5	61.9 ± 0.4
S	15.8 ± 0.1	18.5 ± 0.1	20.2 ± 0.1
G2-M	13.9 ± 0.1	14.3 ± 0.2	17.9 ± 0.2

Mean ± SD

to each of various control and experimental 35-mm dishes, and the final volume adjusted to 2 ml with SFM. The dishes were immediately placed in an incubator at 37 °C in in 8% CO_2. After 24 h the media were aspirated, and SFM containing 0, 85, or 170 n*M* PMA (Sigma) were added to the dishes. The media were then changed every 2 days. At the end of 12 days continuous culture, the cell morphology was observed and classified either into a group comprising bipolar, tripolar and multipolar cells (dendritic cells), or into a group comprising large irregularly shaped cells. The cells were examined under an inverted microscope (Olympus Tokyo, Japan, IMT-2), and the relative proportions of the two groups estimated by counting the cells with the aid of an ocular grid at nine randomly selected positions.

Cell cycle analysis

The fourth-passaged human melanocytes were seeded into plastic dishes (35 mm, Corning) at a density of 5.6×10^4 cells/dish, and the final volume of each dish was adjusted to 2 ml. The cells were incubated in SFM containing 0, 85, or 170 n*M* PMA, and cultured continuously for 12 days without subculture. At the end of 12 days incubation, the cells were rinsed with 2 ml PBS (0.01 *M*, pH 7.2), and the dishes stored at −80 °C after adding 1 ml of 40 m*M* citrate buffer (pH 7.6, containing 250 m*M* sucrose and 5% v/v dimethylsulphoxide) (Merck, Darmstadt, FRG) [16]. For analysis, the frozen cultures were thawed at room temperature and the overlying citrate buffer discarded. Then 0.25% trypsin solution was added to the culture dishes and incubated for 20 min to resuspend the cells. After centrifugation at 500 *g* for 10 min, the cell pellets were resuspended in 0.1% citrate buffer, pH 7.2, and centrifuged again. The supernatant was decanted and 0.3 ml ice-cold propidium iodide (PI) (Sigma) solution (50 µg/ml PI in 0.1% citrate buffer containing 0.125 mg/ml RNAase, Sigma, and 0.1% Triton X-100, Sigma, pH 7.2) was added to each Eppendorf tube containing the cell pellet. The PI-stained cell suspension was kept at 4 °C for 30 min or more, and then at 37 °C for the 15 min immediately prior to cell cycle analysis. The cell cycle was analysed with a fluorescence-activated cell sorter (Coulter EPICS-C) fitted with an argon laser operating at a wavelength of 488 nm.

Direct immunofluorescence examination for HLA-DR and vimentin

Cells were prepared according to the procedure described above. The primary antibodies used were mouse anti-human HLA-DR (diluted 1:20, Becton Dickinson, Sunnyvale, Calif., USA) and mouse anti-human vimentin (diluted 1:40, DAKO, Santa Barbara, Calif., USA). The cells were incubated in primary antibody at room temperature (RT) for 60 min; then incubated in FITC-conjugated goat anti-mouse immunoglobulin (diluted 1:50, Cappel) in the dark at RT for 30 min. Between each step, the cells on the plate were washed with 0.01 *M* PBS, pH 7.2. After mounting in 90% glycerin jelly, the immunofluorescence-stained cells were examined using an immunofluorescence microscope (Nikon UFX-II). The proportion of HLA-DR-positive cells in 200 cells was determined in nine randomly selected fields.

Tyrosinase activity assay

For the assay of the effect of PMA on tyrosinase activity, the cells were seeded at a density of 4.2×10^5 cells/flask. After 24 h, the media were replaced with 4 ml SFM containing 2 µCi/ml ^{3}H-tyrosine and various concentration of PMA (0, 85 and 170 n*M*). After incubation for 24 h, the media were collected for tyrosinase assay. The amount

of ^3H_2O released into the medium when ^{3}H-tyrosine was converted by tyrosinase into 3,4-dihydroxyphenylalanine was measured. From each group, triplicate 4-ml samples of ^{3}H-labelled medium were placed into 15-ml plastic tubes, each of which was treated with 4 ml activated charcoal (Sigma, 20% w/v in 0.2 *N* citric acid) at 4°C for 48 h. After centrifugation (1000 *g*, 10 min), 4 ml of the supernatant from each tube was passed through a Dowex 50 W resin column, and the column rinsed with 2 ml of 0.1 *N* citric acid. Eluent (2 ml) from each column was collected into scintillation vials and counted by a liquid scintillation counter (Minaxi-β Tri-Carb 4000 Series). Tyrosinase activity was expressed as counts per minute, and as percentage of control.

Statistical analysis

Student's *t*-test was used to determine the significance of the results. Triplicate samples from each group of experiments were analysed. Differences with $p < 0.05$ were accepted as significant.

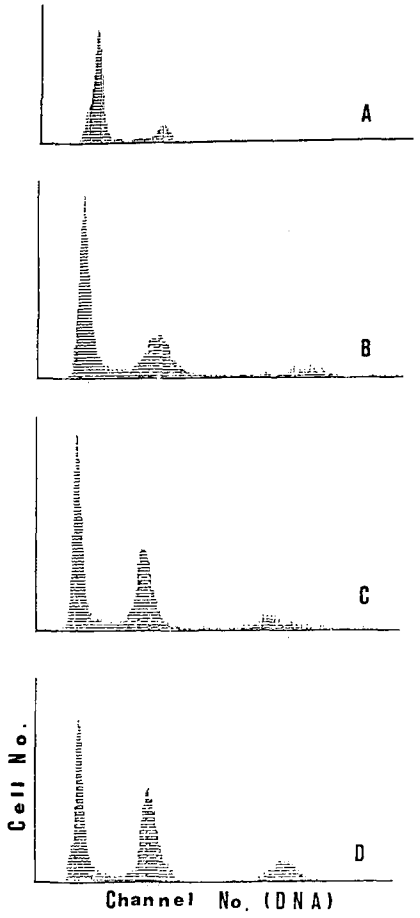

Fig. 3. Cell cycle analysis of melanocytes when cultured in SFM containing various concentration of PMA. **A**, the cell cycle of cells immediately before the experiments: **B**, the cell cycle of cells with 0 n*M* PMA; **C**, the cell cycle of cells with 85 n*M* PMA; and **D**, the cell cycle of cells with 170 n*M* PMA. The higher the PMA concentration in the medium, the higher the percentage of cells in S plus G2-M phase and tetraploid and octaploid karyotype, and the lower the percentage of cells in G0-G1 phase and diploid karyotype

Results

Effects of PMA on morphological changes of melanocytes

When the melanocytes were incubated in SFM and passaged every 5 to 7 days, they appeared either as small bipolar or tripolar cells, or as large multipolar cells.

When the cells were cultured in various concentrations of PMA for 12 days continuously, they could be classified morphologically into a group comprising bipolar, tripolar and multipolar cells and a group comprising large irregularly shaped cells. The latter group comprised $63.2 \pm 3.1\%$ of the total cells when cultured in 170 n*M* PMA, $43.5 \pm 2.5\%$ with 85 n*M* PMA, and $18.2 \pm 1.2\%$ without PMA (Fig. 1). The higher the PMA concentration, the higher the yield of the large irregularly shaped cells. The size of the large irregularly shaped cells was about three to ten times that of the small bipolar cells cultured without PMA. Some of the large irregularly shaped cells resembled atypical multinuclear giant cells (Fig. 1) having more than one nucleus, prominent nucleoli, and abundant cytoplasm.

The cytoskeleton (vimentin) of the large irregularly shaped cells was disaggregated and even lost in the peripheral edges of the cells (Fig. 2). In contrast, the dendritic cells showed compact vimentin within cells.

Cell cycle analysis

The melanocytes incubated in SFM without PMA were subcultured every 5 to 7 days. Part of the first, second and third passaged cells were collected separately for cell cycle analysis. The results are shown in Table 1.

After 12 days culture in SFM containing various concentrations of PMA, the cells were collected for cell cycle analysis. The results clearly show that the higher the PMA concentration, the greater the proportion of cells in G2-

Table 2. Analysis of the DNA content of melanocytes cultured in triplicate in various concentrations of PMA continuously for 12 days. The higher the PMA concentration, the higher the percentage of cells in octaploid or tetraploid karyotypes. Differences compared with the controls (0 n*M* PMA) with $P < 0.05$ considered significant

	170 n*M* PMA (%)	*P*	85 n*M* PMA (%)	*P*	0 n*M* PMA (%)
Diploid	35.8 ± 0.3	< 0.05	40.8 ± 0.3	< 0.05	47.6 ± 0.4
Tetraploid	38.4 ± 0.3	< 0.05	31.8 ± 0.2	< 0.05	28.1 ± 0.2
Octaploid	14.8 ± 0.1	< 0.05	10.2 ± 0.1	< 0.05	6.6 ± 0.1

Mean $\pm$ SD

M phase (0 n*M* PMA, 32.4 ± 0.3%; 85 n*M* PMA, 39.5 ± 0.3%; 170 n*M* PMA, 45.7 ± 0.5%) and S phase (0 n*M* PMA, 11.3 ± 0.1%; 85 n*M* PMA, 13.2 ± 0.2%; 170 n*M*, 15.8 ± 0.3%), and the smaller the proportion of cells in the G0 – G1 phase (0 n*M* PMA; 56.3 ± 0.5%; 85 n*M* PMA; 47.3 ± 0.3%; 170 n*M* PMA, 38.5 ± 0.3%) (Fig. 3). The ratio of cells in diploid, tetraploid and octaploid karyotype were also analysed (Table 2), and the results suggest that the higher the PMA concentration, the higher the percentage of cells in octaploid or tetraploid karyotype, and the lower the percentage of cells in diploid karyotype.

Expression of HLA-DR on melanocytes

HLA-DR was expressed on a minor proportion of the melanocytes growing in PMA (85 n*M*, 3.4 ± 0.4% of total cells; 170 n*M*, 3.2 ± 0.4% of total cells). None of the cells expressed HLA-DR when incubated without PMA. The HLA-DR-positive cells included both large dendritic cells and irregularly shaped cells (Fig. 4).

Effect of PMA on tyrosinase activity

The tyrosinase activity was expressed as a percentage of that in controls (85 n*M* PMA, 157 ± 14%; 170 n*M*, 112 ± 9%). Thus PMA significantly stimulated the tyrosinase activity at the concentration of 85 n*M*, but not at 170 n*M*.

Discussion

Normal human melanocytes in vivo appear as multipolar dendritic cells and express none of the surface antigens associated with melanoma cells [14, 19]. However, under tissue culture conditions supporting vigorous cell growth, melanocytes appear as bipolar or multipolar cells [2, 4, 8, 18] and express most major melanoma-associated antigens such as nerve growth factor receptor, proteoglycan, transferrin-related Mr 97000 protein antigen, Mr 120000 protein, and gangliosides 9-*O*-acetyl GD3 [7]. In this study, the fourth-passaged melanocytes were cultured continuously for 12 days without passage, and were evaluated for the effects of PMA on cellular biology. The proportion of large irregularly shaped melanocytes and their size, abundance of cytoplasm, number of nuclei, prominence of nucleoli, alteration of cytoskeleton and expression of HLA-DR were directly related to the concentration of PMA in the culture medium. An abnormally high percentage of cells in octaploid karyotype was also associated with PMA in the culture medium, with the higher percentage being associated with the higher concentration. In addition, PMA also altered the cell cycle and tyrosinase activity in vitro. All the above results clearly show that PMA alters the model of cell differentiation, and that the higher the PMA concentration in the medium, the greater the degree of alteration of cell differentiation.

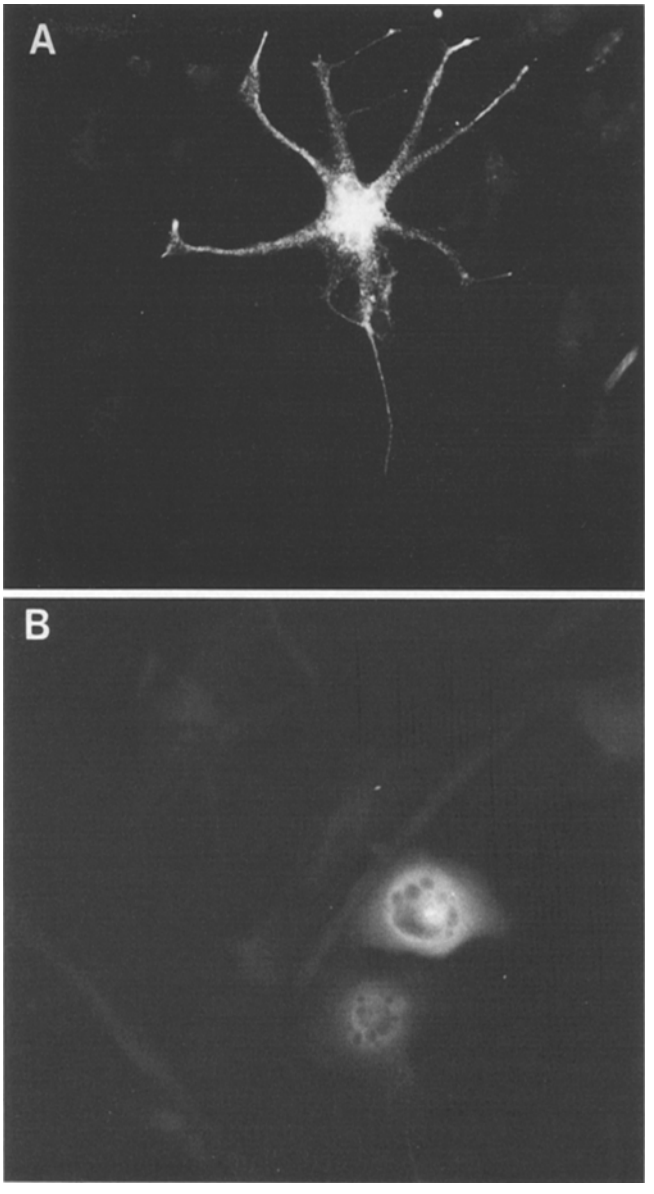

Fig. 4 A, B. HLA-DR direct immunofluorescence examination. Only some large melanocytes expressed the HLA-DR antigens, and most of the cells showed negative staining when growing in SFM containing 170 n*M* PMA continuously for 12 days without passage. Both the large dendritic cells (**A**) and large irregularly shaped cells (**B**) showed positive HLA-DR expression. Direct immunofluorescence, ×400

PMA showed a dose-dependent stimulatory effect on cell proliferation. PMA has been reported to be able to foster replication by permitting the preferential attachment of melanocytes from skin cell suspension and stimulating them to grow [2]. PMA is well known as a classical phorbol diester promoter, which induces various morphological, physiological and biochemical changes in keratinocytes [3, 10, 11]. It is suggested that PMA works through Ca^{2+} and protein kinase C, and the latter apparently possesses multifunctional activity [13]. The mechanism of tumour promotion relating to PMA is not fully understood at present, but the cell differentiation as altered by PMA was observed in some of the cultured melanocytes in this study.

There remains another question as to why the large irregularly shaped cells were not observed when cultured in SFM and regularly passaged at interval of 5 to 7 days. It is proposed that those cells with a tendency to become atypical may attach poorly to plastic flasks or dishes, and they are, therefore, screened out during the passaging procedure. However, it cannot be said that these cells have degenerated and cannot proliferate, because the group with the largest population of large irregularly shaped cells (170 n*M* PMA, 63.2 ± 3.1%) also showed the highest ratio of cells in S and G2-M phase (170 n*M*; S phase, 15.8 ± 3%; G2-M phase, 45.7 ± 0.5%).

In conclusion, when melanocytes are continuously grown for 12 days without passage, evident cell alteration is expressed on some of the cultured cells as changes in morphology, cytoskeleton, cycle and HLA-DR marker. It is shown that the higher the PMA concentration in the culture medium, the higher the degree of altered differentiation. In addition, PMA also affects the tyrosinase activity in vitro. Although the melanocytes growing in media containing PMA still usual appear as bipolar or multipolar in shape, they may be biochemically or physiologically somewhat different compared with those cultured in the absence of PMA. It is proposed that the model of cell differentiation should be altered if the cells are incubated in a medium containing a significant concentration of PMA. Previous reports concerning melanocyte culture systems mention little about the possible alteration in cell differentiation in the presence of PMA [2, 4, 8 18]. Thus, it is recommended that, for further biological studies of melanocytes in vitro, a PMA-free culture system, such as the SFM used in this study, should be used in preference to other systems containing PMA.

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