Basic fibroblast growth factor, bovine pituitary extract, and insulin are essential mitogens for secondary epidermal human melanocyte culture in a serum free medium

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Key words: Cell culture - normal human epidermal melanocytes – growth factors

Abstract

Background: Establishment of a culture system for selective cultivation of normal human melanocytes (NHM) and the identification of the regulatory factors that control their growth is a prerequisite for better understanding of the pigmentary system.

Objectives: Cultivation of NHM in a serum and tumor promoting factor free medium and examination of the effect of different mitogens on NHM proliferation and antigen expression.

Methods: NHMs from new born and adult skin were grown in a serum free medium that consisted of modified MCDB supplemented with cholera toxin, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin, crude bovine pituitary extract (BPE), Ca++, transferrin, hydrocortisone and triiodothyronine. Proliferation rates were assessed in the absence of each supplement every 3 days for 3 weeks. Immunohistochemical labeling (APAAP technique) was done to examine antigen expression by NHM.

Results: Pure NHM could be obtained after almost 4 weeks and could be passaged up to 5 times. Absence of each supplement did not substantially affect NHM morphology. Proliferation rate of secondary NHM was significantly decreased in the absence of bFGF, insulin and BPE as assessed 3 and 6 days of incubation ($p<0.01$). From day 9 through day 21, absence of each supplement was also associated with a significant decrease of proliferation rate ($p<0.001$). NHMs incubated with plain medium were almost absent from culture plates on day 6 and afterwards ($p<0.001$). No evidence of contamination by epidermal or dermal cells was detected as all cultivated cells were labeled with the NHM specific MoAbs HMB45 and K.1.2.58.

Conclusions: From the above data, it is concluded that NHM can be selectively grown in a serum and tumor promoter free medium and that bFGF, insulin and BPE are essential for NHM proliferation from the early phases of their growth. This system provides an important tool in the in vitro investigation of epidermal melanocytes in normal and diseased human skin and the examination of the different mitogens that may influence their proliferation.

Introduction

Normal human melanocyte (NHM) grows poorly in culture unless stimulated by serum and several other mitogens. In 1982, Eisinger and Marko successfully isolated pure NHM in a medium that has been supplemented with 5% fetal bovine serum, the tumor promoter 12-0-tetradecanoylphorbol 13-acetate (TPA) and cholera toxin [1]. Likewise, Tsuji and Karasek obtained NHM in a medium containing in addition to 10% fetal calf serum, cholera toxin, cAMP elevator and 5-fuorouracil[2]. The elimination of Keratinocytes from these cultures has been attributed to the toxic effects of TPA and the cytotoxic drug. Nevertheless, because of the high concentration of serum fibroblasts were still a major contaminant [3]. Furthermore, another major disadvantage of both culture models was the use of serum and potentially mutagenic agents that render obtained cells inappropriate for neither research nor therapy [4]. Gilchrest et al. overcame this problem and successfully isolated NHM in a hormone-supplemented medium that has been enriched with minimal serum supplementation [5]. Nevertheless, keratinocyte and fibroblast elimination and the little yield of NHM were also obvious problems in this culture system. In addition, the characteristics of these culture systems have not been studied in details [3, 5]

This report describes a modification of this culture system, which completely avoids the problem of keratinocyte and fibroblast contamination and supports good melanocyte proliferation in the absence of tumor precursors and without serum supplementation. In addition, the relative significance of each mitogen on melanocyte proliferation has been examined.

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Material and methods

Melanocyte culture was obtained from newborn foreskin and adult truncal skin, obtained from adults undergoing elective operations (3 different donors each, type IV skin). Specimens are transported to the laboratory in tissue culture medium. The steps of skin processing and obtaining cell suspensions are followed as previously described (5,6). Briefly, deep dermis and subcutaneous fat are cut short (to minimize the risk of contaminating melanocyte cultures with fibroblasts), remaining skin is cut into 4-5 mm3 pieces and incubated in 0.25% trypsin (Sigma, St Lois, Mo, USA) for 16 h at 4 °C. During this time, separation of the epidermis from the dermis is observed. Fetal calf serum (FCS) (10%) is added to the specimens to inactivate the trypsin and separation of the epidermis from the dermis is completed with the aid of a forceps. The dermis is discarded whereas the epidermis is vigorously pipetted to obtain a single cell suspension. The epidermal cells are counted in a hemocytometer chamber and inoculated at a density of 1.25 X 105 cells/ml in melanocyte growth medium (MGM) and maintained in 8% CO2 and 92% air. The medium is changed 2-3 times weekly. The complete MGM consisted of modified MCDB 153 (MCDB 153 + amino acids + antibiotics), cholera toxin (10-9 M) (Calbiochem, San Francisco, USA), epidermal growth factor (EGF)(10 ng/ml), basic fibroblast growth factor (bFGF)(2ng/ml) (Boehringer, Mannheim, Germany), insulin (10 µg/ml) (Sigma), crude bovine pituitary extract (BPE)(70 µg/ml) (Clonetics Inc., San Diego, CA, USA), calcium hydrochloride (2mM), transferrin (10 µg/ml) (Sigma), hydrocortisone (1.4x10-6 M) (Serva, Heidelberg, Germany), and triiodothyronine(10-9 M). FCS (2%) was added only during the first 2 days of the primary culture and the first 24 hours of each subculture.

Microscopy

Cells (6-well culture plate) were monitored by inverted phase contrast microscopy (Olympus, Japan). Photographs were taken to document and monitor microscopic alterations after cultures were washed once with Ca++ free phosphate buffer saline (PBS).

Proliferation assays

Second passage pure NHM cultures were used in this experiment (obtained from each of the 3 newborn foreskins). Proliferation assays of cells were carried out (each in triplicate) in tissue culture plates (96 flat bottomed wells) (Becton and Dickenson, Mountain view, CA) by incubating 1x 103 cell/well in 0.2 ml of medium by the highly sensitive fluorimetric microassay using 4-methylumbelliferyl heptanoate. Fluorimetric values, expressed as absolute fluorescence units, were measured by a titertec Fluoroscan II (Flow Lab Mechenheim, Germany) as previously described (7). Each supplement was omitted from the complete MGM and the proliferation rates were measured one day after inoculation (day 0) and every 3 days afterwards for 3 weeks.

Immunohistochemistry

Second passage NHMs from both newborn and adults were labeled with alkaline phosphatase-antialkaline phosphatase (APAAP) technique as previously described (8,9). Briefly, cells were fixed with pre-cooled methanol (-4 C) for 5 min and were exposed to primary antibody (30 min) diluted in 0.1 M (1:100). As a secondary antibody, anti-mouse IgG (Immunotech, Marsielle, France) diluted in 1:100 in PBS was used with APAAP complex (Dako, Glostrup, Denmark). The incubation steps with the secondary antibody and the APAAP complex were repeated twice to intensify the labeling reaction. Naphthol AS BI sodium salt (Sigma, St Louis, USA) and new fuchsin (Merck, Darmstadt, Germany) were used for visualization. Lastly, counterstaining with Mayer's hemalaun sol (Merck) was done. The slides were mounted with Kaiser's glycerol gelatin (Merck) and examined by light microscopy. Fixed cells not exposed to the primary antibody served as negative control. Positive staining appears bright red, whereas negative staining acquires the counterstain as faint blue color.

Statistical analysis

The mean and SD values obtained from the three newborn foreskins (each in triplicate) were calculated and the percentage decrease of proliferation was determined. Student's t test was used for statistical evaluation and p values of < 0.05 or less were considered significant.

Results

1. Morphological features

Microscopic examination of the culture immediately after inoculation of the desegregated epidermal cells revealed rounded refractile cells floating in the medium. However, as early as 12 h of inoculation two populations of cells, namely small dark bipolar and tripolar cells were seen in between flat small rounded and polygonal cells that represent the keratinocytes (Fig,1). On

subsequent days, large sheets of keratinocytes are formed and the number of dendritic cells progressively increased. The presumed dendritic melanocytes were almost always seen in contact with adjacent keratinocytes via their dendrites [5, 10]. After 3-4 weeks in adult culture and 4-5 weeks in newborn culture, ketratinocytes detach from the culture plate and the dendritic cells change their morphology to bipolar or fibroblasts-like spindle shaped cells reminiscent of histopathological feature of intradermal melanocytic nevus (Fig, 2). By the 5th week, almost all keratinocytes have been detached from the culture plate and only one population of cells remained. The presumed melanocytes could be passaged up to 6 times in newborn and 4 times in adult skin (average 5 weeks), after which cells undergo senescence, become clumped and degenerated regardless of any mitogen, including serum, added to them (Fig.3). No striking difference between melanocytes of adults and newborns was observed, though newborn NHM were more slender and tended to be less dendritic. Removal of each mitogen did not have any striking effect on melanocyte morphology as cells retained their characteristic fibroblast-like shape that is seen with the complete MGM, though the density of NHM decreased progressively in the absence of mitogens. NHM incubated with plain medium were almost absent from culture plates from day 6 through day 21 of incubation.

2. Proliferation assays

Proliferation assay in the absence of each mitogen one day after inoculation (day 0) revealed no significant results not only with the complete MGM medium but also with cells incubated with plain medium ($p>0.05$). Interestingly, 3 days after incubation, a significant increase of growth of NHM incubated not only with the complete MGM medium but also with the plain and supplement deficient media when compared with day 0 was detected (<0.001). Absence of cholera toxin, EGF, Ca++, transferrin, hydrocortisone, and triiodothyronine did not significantly affect NHM proliferation ($p>0.05$). However, absence of bFGF, BPE and insulin was associated with a significant NHM growth retardation ($p<0.01$). Similarly, NHM incubated with plain medium showed a significant decrease of proliferation ($p<0.001$). Six days after incubation revealed similar results, though there was a significant drop of proliferation rate values that reached almost less than 50% of day 3 ($p<0.001$). Proliferation rate of NHM on subsequent days, i.e., 9, 12, 15, 18 and 21 revealed significant decrease of proliferation in the absence of each mitogen when compared with the complete MGM ($p<0.001$) (Table 2). From day 12 through day 21, no further significant increase of proliferation rate of the NHM incubated with the complete MGM was detected as the proliferation rate reached to a plateau. Virtually, no NHM proliferation was detected in culture plates incubated with plain medium on day 6 and thereafter (Fig.4).

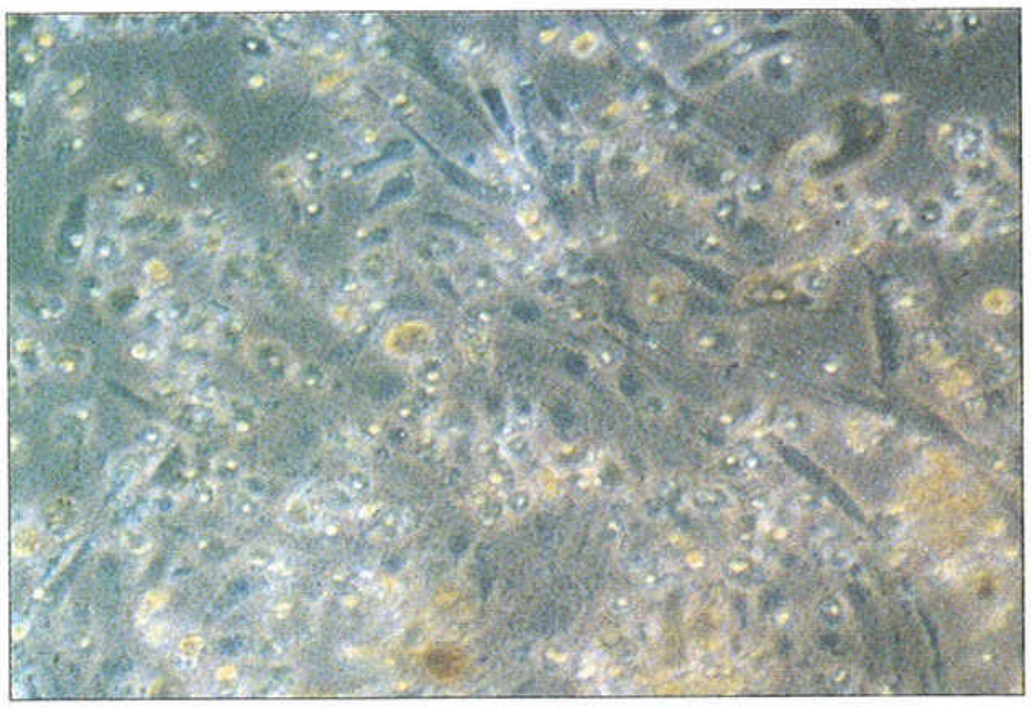
3. Immunohistochemical findings

All cultured cells showed strong positive reaction to MoAbs specific for differentiated melanocytes and melanin producing cells, namely HMB 45 and K.1.2.58. No significant number of cells showing negative labeling was detected. The characteristic fibroblast-like morphology was well preserved and could be easily visualized. The cytoplasm of the cells contained fine granules showing the strong labeling, which represents the labeling with the melanosomal antigen targeted by HMB-45 [11] (Fig. 5a). Similarly, all cultured cells showed negative reaction to MoAbs specific for other epidermal cells, such as basal or suprabasal keratinocytes and Langerhan's cells (Fig. 5b). No difference in antigen expression was detected between newborn and adult skin NHM. Similarly, no striking difference in antigens expression was detected in the absence of any mitogen.

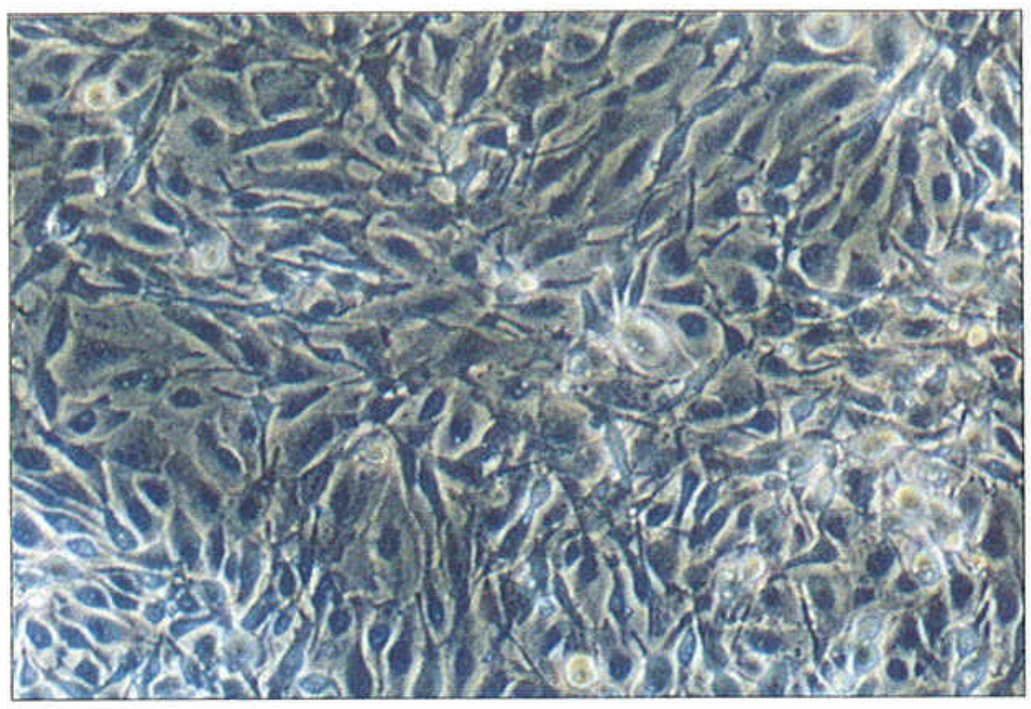
Discussion

Serum is a potent growth factor for all cells and melanocytes are no exception. However, because of its presence, obtained melanocytes may not be optimal for examination of factors that may influence melanocyte function. This is because serum may contain substances having structural or functional similarities with those being tested and hence their effects could be masked. Also, because of the presence of TPA, a potential carcinogenic agent, obtained melanocytes are not suitable for therapeutic purposes (4, 12). Results of our study show that melanocytes, indeed, can be easily grown in a serum- and TPA- free medium.

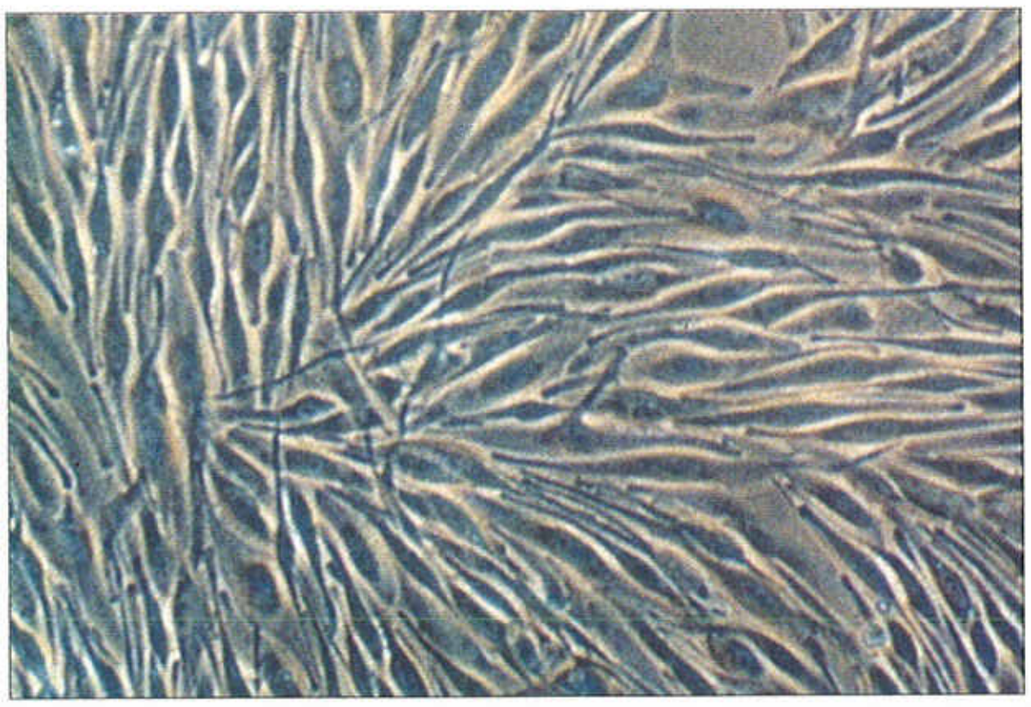
Fetal calf serum (2%) is added to cultures at time of inoculation for 48 hrs in primary culture and with every passage for first 24 hrs only. Addition of serum has been found to be responsible for attachment of the cultivated cells to culture plates and for stimulation of their growth[5]. Although serum was essential for attachment of adult melanocytes, it was not essential for attachment of melanocytes of newborn origin, however, melanocyte yield in the absence of serum was low (20-30%



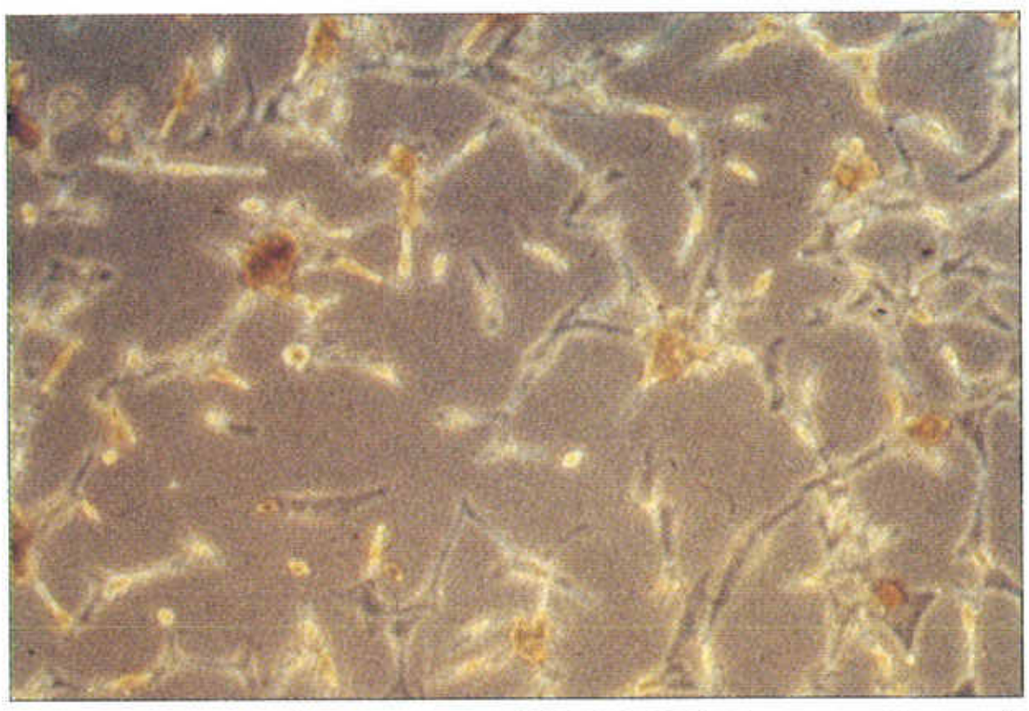
(Fig. 1): Twelve hours after inoculation of the primary culture. Polygonal or rounded and small dendritic cells are seen amid refractile floating cells. The dendritic cells are always seen attached to the polygonal rounded shaped keratinocytes via dendrites (Original magnification, X200)



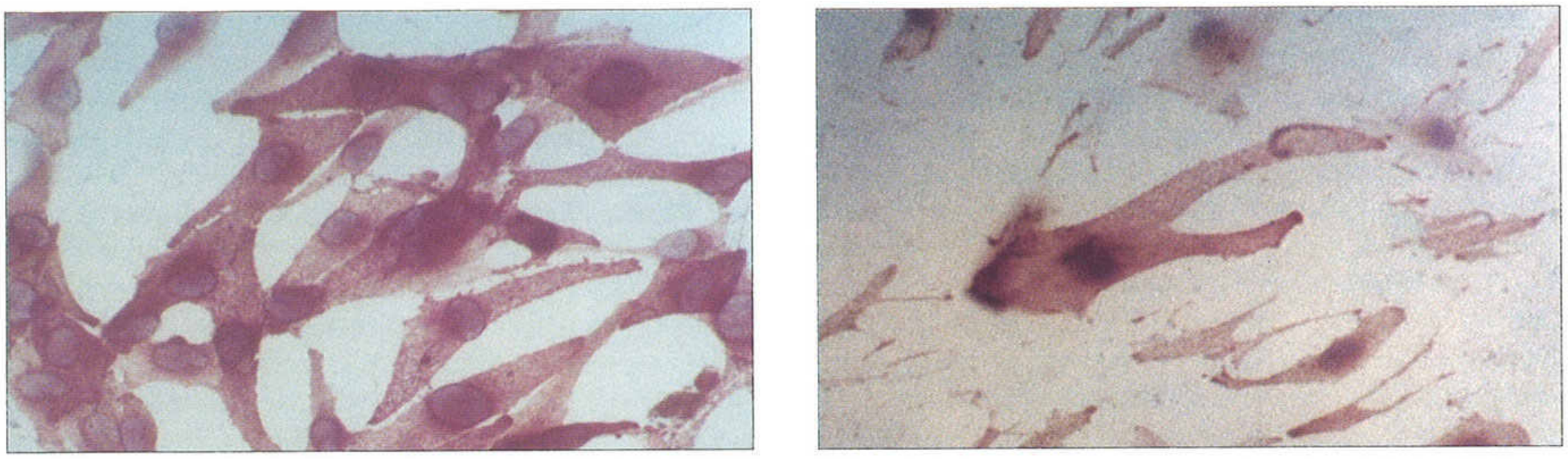
(Fig. 2):Fibroblast shaped NHM clustering around the few remaining keratining keratinocytes and attached to them with their dendrites (Original magnification, X200)



(Fig. 2): Pure NHM in culture are usually slender and fibroblast-like cells. Mitotic figures are commonly seen. (Original magnification, X250).



(Fig. 4): Evidence of senescence of NHM. Cells become clumped with short dendrites, loose proliferation capacity and finally disappear from the culture plate. (Original magnification, X200). .



(Fig. 6): All cultured cells are HMB45+ confirming the identity of these cells (a), whereas other epidermal cell markers failed to label the cells (b). The prominent granular cytoplasm reflects the melanosomal labeling (Original magnification, X250)

of the 2% serum-containing complete MGM medium) (data not shown). Keratinocytes and melanocytes grow usually together, however, after the differentiation, full keratinization and separation, the melanocytes continue to grow and pure melanocyte cultures are obtained. The minor differences of NHM morphology between adults and newborns are most probably due to the relative higher proliferative capacity of the latter.

A constant observation in this study and others is the close relationship of NHM and keratinocytes in which NHMs were almost always seen in contact with the adjacent keratinocyte colonies with dendritic projections. Pure melanocytes in primary and secondary cultures were fibroblast like or spindle shaped cells. Removal of each mitogen did not have any striking effect on melanocyte morphology. Indeed, it has been shown that contact with differentiated keratinocytes is the signal for dendrite formation [10, 13]. Although pure melanocytes could be obtained after several weeks, the presence of keratinocytes was of benefit due to the supporting nature and the paracrine stimulatory effects of these cells [10, 14]. This was also the rational for the use of MCD153 medium with added supplements as it supports growth of both melanocytes and keratinocytes but is suboptimal for fibroblasts [15]. Calcium is usually added to medium to induce keratinocyte differentiation and keratinization rather than as a mitogen [16].

Proliferation assay in the absence of each mitogen revealed significant results that could be detected as early as 3 days of incubation. The initial increase of proliferation that was observed is most likely due to the effect of serum that has been removed on day 0 of assessment. In favor of this assumption is the drastic drop of proliferation on day 6; by that time the effect of serum has disappeared. Nevertheless, absence of bFGF, insulin and BPE resulted in a significant decreased NHM proliferation that was also observed on day 6. As most experimental or therapeutic works are done within one week of NHM passage, it is evident that they are the most significant mitogens [17-19]. Absence of all added supplements revealed significant NHM growth retardation from day 9 and afterwards, indicating that long term NHM cultures require several mitogens for survival and proliferation. It has been shown that confluence or growth factors depletion caused reversible, G1 phase-specific, NHM cycle growth arrest, thus providing an explanation for the almost non significant change in NHM proliferation from day 9 through day 21 of incubation [19]. The main mitogenic component in BPE is bFGF [20]; our results, however, indicate that factors in pituitary extract other than bFGF are also essential for melanocyte proliferation, alpha MSH is a possible candidate [12].

The identity of the melanocytes is confirmed by the positive labeling with specific MoAbs. NHMs in culture but not in situ express melanoma associated antigens. Nevertheless, this phenomenon does not reflect the genetic and biologic properties, since the cultured NHMs have a diploid karyotype, nontumorogenic and do not spontaneously transform in culture [21]. The almost complete absence of other epidermal and dermal cells as shown immunohistochemically is a clear advantage of this culture system.

From the above data, it is obvious that NHM can be selectively cultivated in a serum free medium and without the use of tumor promoters. It is also concluded that bFGF, cholera toxin and BPE are essential for melanocyte growth from the early phases of secondary melanocyte culture, whereas the other supplements, although stimulate melanocyte proliferation are not essential. It is also recommended that examining factors that may influence melanocyte proliferation should be carried out in the absence of all or any of these mitogens according to experimental design.

Table. 1. Specificity of monoclonal antibodies and dilutions used.

MAbs	Specificity	Source	Dilution
K.1.2.58	Early melanoma marker	Zelldiagnostika, Munster, Germany	1:20
HMB 45	Activation marker	Enzo, NY, USA	1:6000
K1	CK1(suprabasal keratinocytes)	Enzo, NY, USA	1:4000
K10	CK10 (suprabasal keratinocytes)	Monosan	1:50
PKK2	CK5, 14-17 (basal keratinocytes)	Labsystem	1:350
BL6	CD1a (Langerhans cells)	Immunotech, Marsielle, France	1:800

Table. 2.

Absolute Fluorescence Units (mean + SD)/ Percentage Decrease of Proliferation

Absent mitogen	Day (0)	Day (3)	Day (6)	Day (9)	Day (12)	Day (15)
Cholera toxin	537.7+18.9/81.5%	860.0+90.4/82.8%	416.8+19.0/102.2%	419.9+12.1/45.5%**	468.2+10.6/44%**	461.7+23.6/43.4%**
EGF	582.4+19.2/88.3%	885.9+69.9/85.3%	421.5+21.4/103.4%	444.9+14.3/48.2%**	579.7+21.3/54.4%**	575.8+14.4/54.1%**
bFGF	590.6+37.3/89.4%	757.9+30.7/72.9%*	329.9+13.6/80.9%*	379.2+08.1/41.1%**	504.2+12.6/47.4%**	510.5+25.0/48%**
Insulin	589.4+13.7/89.3%	814.2+28.6/78.4%*	329.0+20.2/80.7%*	430.2+02.7/46.6%**	500.7+15.0/47.1%**	543.9+11.1/51.1%**
BPE	602.2+22.5/91.3%	835.6+26.2/80.4%*	334.8+22.5/82.2%*	335.3+9.4/36.4%**	360.4+12.5/33.9%**	400.6+16.3/37.7%**
Ca ++	606.2+10.2/91.9%	975.3+76.4/93.9%	399.5+11.5/98.0%	408.1+6.9/44.2%**	466.3+45.3/43.8%**	497.5+29.8/46.7%**
Transferrin	583.7+08.3/88.5%	992.4+82.3/95.5%	414.1+08.9/101.6%	529.7+28.7/57.4%**	595.8+43.3/56%**	705.5+16.3/66.3%**
Hydrocortisone	582.4+13.9/88.3%	974.2+52.0/93.8%	433.8+33.6/106.5%	572.5+13.9/62.1%**	669.6+57.9/62.9%**	727.6+18.2/68.4%**
Triiodothyronin	632.9+42.1/95.9%	928.9+33.8/89.4%	434.5+23.2/106.6%	561.8+20.8/60.9%**	630.4+20.3/59.2%**	715.5+15.0/67.2%**
Plain medium	654.4+26.4/99.2%	741.3+51.7/71.3%**	104.6+4.4/25.7%***	51.3+4.1/5.6%***	114.2+12.9/10.7***	89.4+6.2/ 8.4%***
Complete MGM	659.8+53.9	1039.5+37.6	407.5+2.3	922.4+14.7	1064+14.5	1064+30.4

** P <0.01, ** P<0.001 *** P<0.0001 when values were compared with the complete MGM medium on the same day of incubation.*

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