Egypt, J. Plast. Reconstr. Surg., Vol. 29, No. 1, January: 61-66, 2005

Differential Trypsinization-Selective Adhesion Technique for Cultivation of Normal Adult Human Melanocytes in Phorbol Acetate-Free and Cholera Toxin-Free Medium

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ABSTRACT

Cultured keratinocyte grafts are hypopigmented and the currently used tumor promoter-dependent techniques for cultivation of melanocytes are neither successful for adult cells nor suitable for transplantation. A culture technique was developed that allows rapid multiplication of adult melanocytes in vitro without using the tumor promoter phorbol 12-myristate 13-acetate (PMA) or cyclic adenosine mono-phosphate (cAMP) elevating agents. Melanocytes were separated from mixed keratinocyte/melanocyte primary cultures by differential trypsinization and were isolated by selective adhesion in custom-made, calcium-free, serum-free MCDB-153 (Molecular, Cellular and Developmental Biology). A pure strain of dendritic melanocytes that stain positively with L-3,4 dihydroxyphenyl alanine (L.DOPA) was obtained after shifting into low calcium MCDB-153 supplemented with 2% fetal bovine serum (FBS). Melanocytes proliferated rapidly and were serially passaged every 4-6 days for 8 passages with split ratio of 1:2 to 1:3. MCDB-153 supplemented with either PMA or basic fibroblast growth factor (bFGF) did not support optimal melanocyte growth beyond P2. The low serum, tumor promoter-free and cholera toxin-free adult melanocytes culture technique is simple, acceptable for human use and allows normalization of melanocyte content in cultured epidermal sheets for possible transplantation.

INTRODUCTION

Melanocytes are specialized dendritic cells derived from the neural crest that populate the skin in the intrauterine life and are found scattered between the keratinocytes of the basal layer of the epidermis after birth. Their number varies from region to region of the integument but does not vary with the skin color or the race **[1]**. They produce melanin which determines the skin color and protects the skin against the deleterious effects of the ultraviolet radiation on keratinocyte nuclei and the dermal collagen **[2]**. Normally, melanocytes undergo very little replication in vivo and were considered difficult to grow till the past two decades, when successful techniques for melanocyte cultivation were described **[3-8]**. These techniques utilize modifiers of the cell physiology and/or the tumor promoter PMA. Therefore, they are neither suitable for studying carcinogenesis in melanocytes nor for transplantation to treat hypopigmented skin conditions. In this study, we describe a new technique for cultivation of normal adult human melanocytes and their response to the tumor promoter PMA and the natural mitogen bFGF. The potential clinical application is also described.

MATERIAL AND METHODS

The basic MCDB-153 medium was prepared as described by Boyce and Ham **[9,10]**. It was supplemented with 0.6uM hydrocortisone, 10ng/ml epidermal growth factor, 5ug/ml insulin and 6mg% bovine pituitary extract (BPE). The complete medium contains 0.15mM Ca Cl_2. Calcium was excluded from the custom-made, calcium-free medium prepared by us.

Cultures:

The primary epidermal cell cultures were established from normal adult human skin as described by Marcelo et al. **[11]**. Melanocytes were separated from these mixed primary cultures **[12]**. The remaining keratinocytes were either subcultured or cryopreserved in liquid nitrogen **[13]**. Melanocytes were subcultured either in the complete MCDB-153 supplemented with PMA (10ng/ml) or bFGF (10ng/ml) or in 1:2 mixture of the regular MCDB-153 medium and our calcium-free medium. The later was changed after 24 hours into the calcium-free MCDB-153 medium. The media were changed every other day till the 4^{th}-6^{th} day, when we shifted our calcium-free cultures into the complete, low-calcium, MCDB-153 supplemented with 2% FBS. With all cultures, attempts

at serial subcultivation were made at $1x10^6/cm^2$. Mixed cultures were done by plating P2 melanocytes grown by our technique into pairs of 60 mm culture dishes at $0.5x10^6$, $1x10^6$ and $2x10^6/cm^2$ densities. Cryopreserved P1 keratinocytes were added 24 hours later at a fixed density of $2x10^6/cm^2$ and the cultures were fed the low-calcium, serum-free MCDB-153. After confluency, a representative culture of each pair was stained with DOPA. The optimal melanocyte/keratinocyte ratio was considered the one that can achieve 10-25% melanocytes in the basal layer **[14]**. It was determined by cell counting and scanning under the inverted phase microscope. Keratinocyte differentiation and stratification were induced by adding 1.2mM Ca Cl_2 to the other culture dish.

Microscopic examination:

Melanocyte cultures were examined microscopically with and without DOPA staining, leucine aminopeptidase staining and indirect immunofluorescent staining using Mel-5 antibody to confirm pigment cell selection and to exclude the presence of any significant fibroblastic contamination **[12,15,16]**.

Melanin assay was done and melanin content was assayed by measuring the absorbence of the samples and different dilutions of standard melanin at OD-475 in Beckman DU-600 spectrophotometer **[17]**. Melanocyte growth was assessed by cell counting at the end of each passage, using the standard hemocytometer chamber.

RESULTS

Culture technique:

The cell population separated by differential trypsinization of mixed keratinocyte/melanocyte primary cultures was heterogenous. We estimated the melanocyte content in this mixture by DOPA staining of 3 overnight cultures plated in MCDB-153 medium into grid-style culture dishes. Scanning 3 randomly-selected squares was done under the inverted phase microscope. The average melanocyte content of this mixed cell population was 83.87% (Table 1).

When grown in our calcium-free medium, keratinocytes were excluded while melanocytes remained attached and appeared less dendritic than in the primary cultures. After shifting into the low-calcium, complete MCDB-153 medium supplemented with 2% FBS, they enlarged, became dendritic and proliferated as evidenced by the detection of mitotic figures and increased cell count (Fig. 1). They were identified by their dendritic appearance, the presence of melanin granules and by DOPA and indirect immunofluorescent staining using Mel-5 antibody (Fig. 2). A pure melanocyte strain could be obtained as early as P1 and the presence of significant fibroblastic contamination was excluded by leucine aminopeptidase staining (Fig. 3). They were serially passaged to P5 with split ratio of 1:2 or 1:3 every 4-6 days. They undergo one population doubling every 3-6 days (Table 2). In the late passages, the growth rate slowed down, with gradual decrease in the relative nuclear size and accumulation of vaculated cytoplasm and eventual complete senescence.

Responses to PMA and bFGF:

The tumor promoter PMA could exclude keratinocytes from the melanocyte-enriched cell population separated by differential trypsinization in P1. It could stimulate the proliferation of adult melanocyte for one or two passages. Adult melanocytes grown in PMA-supplemented MCDB-153 were initially dendritic. They turn gradually into bipolar. They were DOPA positive and were arranged in clusters (Fig. 4). They could not be successfully subcultivated beyond P3. Under our experimental conditions, the bFGF showed a weak mitogenic effect on adult melanocytes. Alone, it neither excluded contaminating keratinocytes nor enhanced considerable melanocyte proliferation. Contrary, bFGF exhibited a strong mitogenic effect on contaminating keratinocytes which dominated the cultures by P3.

Melanogenogenic potential of cultured melanocytes:

Melanin assay was done in 7 P2 strains, 48 hours after plating at a density of $1 \times 10^4/cm^2$ in T-25 cm^2 flasks, with an internal standard for each experiment. The amount varied between 8.33-109.69 ug/flask (Table 3). The mean melanin content, excluding case #3, was 13.97 ug/flask. The highest melanin content was observed with the only black skin-donor in this series of cases (case #3).

Melanocytes in cultured epidermal grafts:

By total and differential cell counting and scanning of the mixed confluent cultures stained with DOPA under the inverted phase microscope, the proper melanocyte/keratinocyte ratio that optimized the melanocyte content in cultured epidermis was found to be 1:2. Detachment of the stratified cultured sheets was done by dispase II. Minute holes were observed in the high melanocyte density sheet probably at the areas where melanocytes were clumped in the culture dish.

Fig. (1): P2 melanocytes cultured by our technique. They are dendritic and show mitotic activity (X 100).

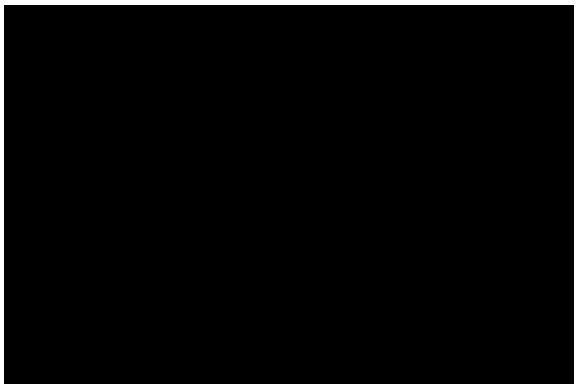

Fig. (2): Cultured P2 melanocytes showing positive indirect immunofluorescent staining using Mel-5 antibody.

Fig. (3): Leucine amino-peptidase histochemical stain, negative for P2 cultured melanocytes (a) and positive for P2 cultured fibroblasts (b).

Fig. (3-A): P2 melanocytes stained negatively with leucine aminopeptidase.

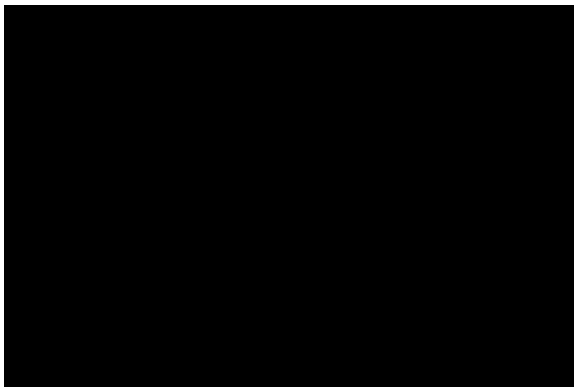

Fig. (3-B): P2 fibroblasts stained positively with leucine aminopeptidase.

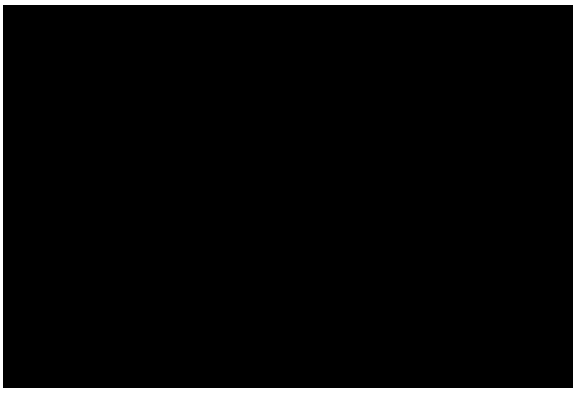

Fig. (4): P2 melanocytes cultured in PMA containing medium (X100). The cells are arranged in clusters and most of them do not retain their typical dendritic appearance.

Table (1): The relative composition of the cell population separated by differential trypsinization.

Case #	Field #	Mel.	Ker.	Total	% Mel.
1	a	49	12		
	b	41	8	156	82.7%
	c	39	7		
2	a	47	8		
	b	43	8	165	83.6%
	c	50	9		
3	a	31	6		
	b	32	5	109	85.3%
	c	30	5		
Mean					83.3%

Differential melanocyte/keratinocyte count done on 3 different microscopic fields (a,b&c) in 3 different cell strains (1,2&3), stained with DOPA after an overnight incubation.

Table (2): The results of melanocyte growth by differential-trypsinization, selective-adhesion culture technique.

Case #	P1	P2	P3	P4	P5
1	0.44	0.39	0.56	0.56	0.42
2	0.42	0.30	0.72	0.75	0.50
3	0.34	0.55	0.75	0.60	0.50
Mean	0.40	0.41	0.68	0.64	0.47
PD	0.67	0.70	1.43	1.36	0.90
PDT	142.8	137.1	67.1	70.6	106.7

The population doubling (PD) and the population doubling time (PDT), were calculated based on the average cell yeild/flask in each passage, according to the following formulas:

$PD = (\log N_H - N_I) / \log 2$

$PDT = (t_2 - t_1) / PD$

Where N_H is the number of cells harvested, N_I is the number of cells inoculated, t_2 is the time at which the cells were harvested and t_1 is the time when the cells were inoculated (t_1 = zero).

Table (3): Melanogenic power of P2 cultured melanocytes.

Case #	Melanin ug/flask
1	8.33
2	9.66
3	109.69
4	17.48
5	20.16
6	18.99
7	9.21
Mean* (n=6)	13.97

* The mean calculated after excluding the only black skin donor in this series.

DISCUSSION

In vitro cultivation of normal human melanocytes has to overcome the presence of fibroblasts and keratinocytes that grow faster than melanocytes and the slow division rate of melanocytes **[3]**. Fibroblasts were dealt with by the antibiotic geneticin 18 or by the physical separation of the epidermis from the dermis **[18,7]**. Keratinocytes were excluded by PMA, 5-fluorouracil, or the use of dialized hypothalamic extract that lacks the low molecular weight keratinocyte growth factor **[3,19,4]**. In order to enhance melanocyte proliferation, a variety of mitogens were used. Most of the melanocyte culture techniques utilize the tumor promoter PMA either alone, or in combination with cAMP elevating agents **[3,5,7]**. In other techniques, tissue extracts were used **[4,5,15]**. A major advance was made when bFGF was identified as the natural melanocyte mitogen that could replace PMA in cultures of normal human melanocytes **[6,20]**. In our PMA-free, cholera toxin-free melanocyte culture technique, fibroblasts were excluded both by mechanical separation of the epidermis from the dermis and by the low-calcium, serum-free primary culture conditions. Subsequent addition of 2% FBS was suboptimal for contaminating fibroblast as confirmed by leucine aminopeptidase staining. Keratinocytes were excluded by the differential trypsinization and by calcium deprivation for 4-6 days. The low-calcium concentrations did not support continued keratinocyte attachment or colony formation **[21,10]**. Without calcium deprivation, the few keratinocytes seperated with the melanocytes would grow and dominate the culture.

Positive identification of pigment cells within the cultures assumes an importance because of the heterogenous cell populations in the dispersion culture technique **[22]**. The cell-identity was confirmed by both DOPA staining and indirect immunofluorescent staining using the monoclonal Mel-5 antibody. This antibody probably binds to one of the structural proteins of the melanosome **[23]**.

Melanin content correlates with tyrosinase-activity. Melanogenesis varies with race and genetic constitution, being more in black **[24,17]**. Melanocytes cultured by our technique retain their melanogenic power, indicating that the culture system provides the signals necessary for melanogenesis **[4]**.

Most of the melanocyte culture techniques utilize various proportions of different sera. Serum is a potent stimulus for human melanocytes **[25]**. It contains EGF, bFGF, PDGF, IGF, IL1, IL6 as growth factors beside polyamines and trace elements **[26]**. It was observed that 0.5-1% FBS enhanced the growth of melanocytes and that they did not observe any growth of adult melanocytes without 1-2% serum **[4]**. Normal melanocytes were found to require specific growth factors in addition to those supplied in serum **[6]**. In our culture system, FBS at 2% concentration is the only source of mitogenic stimulus we used to supplement the complete MCDB-153 medium for serial subcultivation of adult melanocytes. Complete MCDB-153 culture medium contains bFGF, a major mitogen present in BPE **[6,27,28]**. Failure to get satisfactory growth-response with PMA and bFGF could be explained by the absence of cAMP elevating agent **[6,29]**. However, Pittelkow and Shipley had the same result with adult melanocytes grown in serum-free MCDB-153 supplemented with PMA whether cholera toxin was added or not **[7]**. Loss

of the highly labile cAMP stimulatory activity of bFGF, or an inhibitory effect on melanocyte proliferation with the dose used in our experiment, coupled with its strong keratinocyte mitogenic activity are possible explanations for the poor melanocyte growth-response **[27,30,31]**. The undefined and variable compositions of both FBS and BPE are definite drawbacks in our culture technique. However, normal adult melanocytes grown by this technique are responsive to stimuli, overcoming a major drawback of the cAMP/PMA dependent culture techniques **[29]**.

The behavior of melanocytes in cultures was subjected to extensive research. When grown in vitro, they have an altered morphology which is different from the dendritic appearance observed in vivo and produce less melanin in response to mitogenic stimuli **[25]**. This was attributed in part to their proliferation and in part to absence of various signals provided by the surrounding keratinocytes or received from the environment, such as UV radiation **[31]**.

Success with cultured epidermal-cell grafting encouraged the utilization of the cultured melanocytes and primary epidermal cell culture to treat hypopigmented skin lesions **[16,32,33,34]**. The drawback of the first approach was the transplantation of possibly initiated cells grown in the presence of the tumor promoter while the second approach did not permit expansion of melanocyte-cell population for coverage of large body surface areas. More recently, suspensions of cultured melanocytes and sheets of subcultured epidermal cells were described to cover more extensive areas of hypopigmentation **[35,36]**. The first technique is time consuming and cultured epidermal grafts produced by the second technique are hypopigmented. We believe that optimization of melanocyte content of cultured epidermal grafts by the method we described is both safe and potentially effective mean for transplanting autologous adult human melanocytes onto extensive areas of hypopigmentation.

Summary:

We described a new PMA-free, cholera toxin-free technique for cultivation of normal human adult melanocytes. It is simple and would be more sensitive to physiological stimuli used in pigment cell research. The optimization of melanocyte content of cultured epidermal graft is expected to be of both aesthetic and protective value. We suggest the use of melanocytes cultured by this technique to treat hypopigmented skin lesions.

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