Culture of Melanocytes Obtained from Normal and Vitiligo Subjects

Sungbin Im\textsuperscript{1}, Seung Kyung Hann\textsuperscript{1}, Yoon-Kee Park\textsuperscript{1} and Hyung Il Kim\textsuperscript{2}

The development of human melanocyte culture in vitro from normal adult skin and uninvolved skin of vitiligo patients is essential to investigate the mechanism of depigmentation in vitiligo and other pigmentary dermatoses. By using selective growth and long-term maintenance conditions, we selectively cultured melanocytes derived from normal foreskins and arm skins, and uninvolved foreskins and arm skins of vitiligo patients. The melanocytes of the arm skins were successfully cultured from the roofs of suction blisters. Melanocyte Growth Media (MGM) consisting of MCDB-153 formulation with basic fibroblast growth factor (bFGF), bovine pituitary extract (BPE), insulin, hydrocortisone, phorbol 12-myristate 13-acetate (PMA) and 10% human AB serum was sufficient to grow the melanocytes from normal and vitiligo donors. Melanocytes from uninvolved skin of vitiligo donors showed no different morphologic features, initial seeding capacity and population doubling time compared with those from normal skin. Melanocytes from both cell types grew without any lag period for more than 6 months (6-11 passages). Melanocytes obtained from foreskins had higher initial seeding capacity and shorter population doubling time than those obtained from arm skins using suction-blistered roofs. Our results suggest that the culture method using suction blisters may be a simple and easy way to obtain melanocytes. In addition, vitiligo melanocytes can be successfully cultured with appropriate growth conditions and may show no defective growth patterns. This culture system will be applied to investigate the basic pathophysiology of vitiligo and other various pigmentary dermatoses.

\textbf{Key Words:} Culture of melanocytes, vitiligo

Vitiligo is an acquired pigmentary disorder characterized by circumscribed patchy depigmentation of melanocytes (Ortonne et al. 1983, Hann et al. 1986). Its etiology is not clear, and to date many efforts have been focused on the neurohormonal theory, autodestruction theory, autoimmune theory (Naughton et al. 1983; Norris et al. 1988; Park et al. 1991) and recently the hereditary defective melanocyte theory (Puri et al. 1987; Boissy et al. 1990).

Puri et al. (1987) reported that cultured melanocytes from individuals with vitiligo manifested defective growth and passage capacity. These defects could be spontaneously corrected in repigmenting subjects and partially corrected by the addition of fibroblast-derived growth factors in vitro (Puri et al. 1989). Recently Medrano and Nordlund (1990) reported that by using new growth conditions, pure melanocyte cultures were successfully obtained from normal adults and the pigmented skins of vitiligo donors without any significant differences in growth characteristics.

In the present study, we described the modified conditions for optimal melanocyte culture from foreskins and suction-blistered roofs of normal adult skin and nonpigmented skin of vitiligo donors. We also showed growth characteristics of both cell types which will be required for subsequent research of vitiligo.

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MATERIALS AND METHODS

Culture reagents

During the culture procedures, Melanocyte Growth Medium (MGM) (Clonetics Corporation, San Diego, CA) was used. This modified serum-free MCDB-153 formulation is supplemented with basic fibroblast growth factor, recombinant (bFGF) (1 ng/mL), bovine pituitary extract (BPE) (0.4% v/v), insulin, bovine (5 μg/mL), hydrocortisone (0.5 μg/mL), phorbo1 12-myristate 13-acetate (PMA) (10 ng/mL) and antimicrobial agents. Heat-inactivated human AB serum was added to the media with the concentration of 10% (v/v). Trypsin (0.25% w/v)/EDTA (0.01% w/v) solution, trypsin neutralizing solution and HEPES buffered saline solution used as subculture reagents were purchased from Clonetics Corporation.

Cell sources

Foreskin melanocytes were obtained by circumcision from 7 normal volunteers and 5 actively spreading vitiligo patients (Table 1). After excision, foreskins were washed with ethanol (75%) for 15 minutes and placed into a sterile container filled with RPMI (4°C). Subcutaneous fat and deep dermis were carefully removed and each specimen was cut into 4 x 4 mm pieces. Each specimen was placed in trypsin/EDTA (0.25%/0.1%) solution at 4°C for 12 hours. The trypsin was then replaced by RPMI and the epidermis of each piece was detached from its underlying dermis using fine forceps. Arm skin melanocytes were obtained by the suction blister method from 5 normal volunteers and 5 actively spreading vitiligo patients (Table 1). The suction blisters were produced on the inner surface of the arm skin using a suction device as described by Hann et al. (1992). The negative pressure was maintained at 200 mmHg for one and half hours.

For each subject, 10 blisters, each 7 mm in diameter, were obtained.

Primary culture

Each epidermal sheet was incubated in trypsin/EDTA (0.25%/0.1%) solution at 37°C for 30 minutes. After incubation, the epidermal sheet suspension was pipetted 20 times and filtered with mesh. The filtered epidermal cell suspension was collected in trypsin neutralizing solution and centrifuged at 180 ×g for 10 minutes and resuspended in MGM.

Cell viability was determined by trypan blue dye exclusion test. Fibroblasts, if present, were removed by geneticin treatment (Halaban and Alfano, 1984). The epidermal cell suspensions from foreskin and from arm skin were seeded in culture vessels at a density of 2 x 10⁵ cells/cm². They were incubated at 37°C in humidified atmosphere containing 5% CO₂. The media were changed after 2 days and every 3 days there after.

Cell counting

Adult melanocytes were easily identified by their dendritic processes and in situ counts of at least 10 randomly selected microscopic fields (0.49 mm²) were quantified visually according to the method of Aubock et al. (1983).

Cell passage

Upon reaching subconfluency, the culture medium was aseptically removed from culture dishes and washed with HEPES buffered saline and then with trypsin/EDTA (0.25%/0.01%). When the melanocytes became preferentially detached, trypsin neutralizing solution was added. The melanocytes were collected in MGM and replated at a density of approximately 5 x 10⁵ cells/cm². Keratinocytes, if present, were selectively removed by differential trypsinization.

Experimental design

Experiments were performed to examine some characteristics of this melanocyte culture system: (1) the optimal growth conditions of melanocytes from normal and vitiligo donors; (2) the morphologic characteristics of melanocytes obtained from foreskin and arm skin; (3) the morphologic characteristics of melanocytes obtained from normal and vitiligo donors; (4) the differences of initial seeding capacity between normal and vitiligo melanocytes; (5) the differences in growth rate between two cell types; and (6) life span of both cell types.

<table>
<thead>
<tr>
<th>Table 1. Profiles of subjects</th>
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<tr>
<td>No. of subjects</td>
</tr>
<tr>
<td>Normal arm skin</td>
</tr>
<tr>
<td>Vitiligo arm skin</td>
</tr>
<tr>
<td>Normal foreskin</td>
</tr>
<tr>
<td>Vitiligo foreskin</td>
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To examine the optimal growth conditions, the relationship between incubation time of the epidermal sheet and viability of epidermal cell suspension were evaluated. The effect of human AB serum was evaluated and the ability of passage of melanocytes was observed. The morphologic patterns of cultured melanocytes were compared using phase contrast microscopic examinations and photographed daily. Initial seeding capacity was defined as the number of melanocytes per microscopic field (0.49 mm²) 48 hours after seeding 10⁶ epidermal cells (Puri et al. 1987), and evaluated for each cell type. Population doubling time (PDT) is defined as the time interval of one population doubling and calculated from the formula \( PD = 3.33 \times \log_{10} (N/N_0) \), where \( N = \) cell number at harvest and \( N_0 = \) cell number inoculated. This was observed after first passage. Each cell type was passed in sub-confluence state and maintained to its senescence.

RESULTS

A total of 24 primary cultures of melanocytes were successfully kept on longterm maintenance (Table 1). All of these primary cultures were performed using the medium and conditions described in MATERIALS AND METHODS. From 10 blister roofs (each 7 mm in diameter), 0.7-1.6 \( \times \) 10⁶ free epidermal cells could be obtained.

Single cell suspensions prepared from foreskin or arm skin by our culture methods showed more than 80% viability in both normal and vitiligo donors. Samples with viability less than 60% showed no attachment to culture dishes or failure to grow. After 1 day in culture, dendritic processes were observed and melanocytes could be easily distinguished from keratinocytes and fibroblasts (Fig. 1). Melanocytes were also identified by the Dopa staining method (Fig. 2).

Contamination by keratinocytes, although a rare occurrence, could be easily removed by differential trypsinization. Occasionally fibroblasts were observed in the late period of primary culture; they could be removed by continuous treatment with genicin and differential trypsinization. Human foreskin melanocytes maintained in our culture conditions showed mostly bipolar and occasionally multipolar dendrites and no morphologic differences between normal and vitiligo subjects were observed in the initial stages and later passages (Fig. 3). Although dendritic processes overlapped, the nuclei and cell bodies never showed overlapping. Human arm skin melanocytes were larger than foreskin melanocytes and had multiple dendrites and also exhibited no morphologic differences between normal and vitiligo subjects (Fig. 4).

![Fig. 1. Melanocytes cultured from arm skin of vitiligo subject on day 1. Melanocytes with their prominent cytoplasm and long thin dendrites can be easily distinguished from other epidermal cells (×100).](image-url)
Culture of Melanocytes in Vitiligo Subjects

Omission of human AB serum from culture medium gave rise to the shortening of dendritic processes, swelling of the cytoplasm and eventually differentiation rather than proliferation of the melanocytes. Melanocytes from normal and vitiligo foreskins showed linear growth characteristics and no lag period. Melanocytes from both arm skins also showed similar growth patterns. The number of

Fig. 2. Dopa-positive human uninvolved arm skin melanocytes from vitiligo donor 7 days after first passage. No contamination of fibroblasts was noted (Dopa stain, ×200).

Fig. 3. Eight-day-old primary culture of human uninvolved foreskin melanocytes from vitiligo donor showing mostly bipolar dendritic processes (×100).
melanocytes grew linearly and thus in 14 days of primary culture, we could not count the number due to subconfluence. The average initial seeding capacity of normal foreskins was 80.4 ± 19.9 and that of vitiligo foreskin was 72.4 ± 9.1. The average initial seeding capacity of normal arm skin was 47.4 ± 18.9 and that of vitiligo arm skins was 52.6 ± 8.1 (Table 2). There was no statistically significant difference between normal and vitiligo groups. However, foreskins showed higher capacity of initial seeding than arm skins in both normal and vitiligo groups. Vitiligo foreskins and arm skins could be easily passed without any growth defect. The population doubling times of normal and vitiligo foreskins were 80.4 ± 22.7 and 78.7 ± 17.2 hours respectively, and those of normal and vitiligo arm skins were 92.8 ± 24.7 and 94.6 ± 12.3 (Table 3). There were no statistically significant differences between normal and vitiligo groups but foreskins showed more rapid proliferation rate than arm skins. Melanocytes ob-

![Image](image_url)

**Fig. 4.** Ten-day-old primary culture of human uninvolved arm skin melanocytes from vitiligo donor showing larger cytoplasm and characteristic multipolar dendritic processes (×100).

<table>
<thead>
<tr>
<th>No. of subjects</th>
<th>Normal</th>
<th>Vitiligo</th>
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<tbody>
<tr>
<td></td>
<td>arm skin</td>
<td>foreskin</td>
</tr>
<tr>
<td>2</td>
<td>74</td>
<td>57</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>102</td>
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<td>6</td>
<td>42</td>
<td>92</td>
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<td>7</td>
<td>47</td>
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Mean ± SD: 47.4 ± 18.9°  80.4 ± 19.9°  52.6 ± 8.1°  72.4 ± 9.1°

*Values are expressed as Means ± SD.*

*p < 0.05, as compared with Mann-Whitney test.*
Culture of Melanocytes in Vitiligo Subjects

Table 3. Culture yield and lifespan of melanocytes

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<tr>
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<th>Population doubling time (hr)</th>
<th>No. of passages (mean)</th>
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<tbody>
<tr>
<td>Normal arm skin</td>
<td>92.8 ± 24.7*</td>
<td>7.8 ± 1.5</td>
</tr>
<tr>
<td>Vitiligo arm skin</td>
<td>94.6 ± 12.3</td>
<td>7.0 ± 1.3</td>
</tr>
<tr>
<td>Normal foreskin</td>
<td>80.4 ± 22.7*</td>
<td>10.4 ± 1.5</td>
</tr>
<tr>
<td>Vitiligo foreskin</td>
<td>78.7 ± 17.2</td>
<td>11.6 ± 1.1</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD.
* p < 0.05, as compared with Mann-Whitney test.

tained from normal and vitiligo subjects were successfully passaged up to 13 times with a 1:2 split ratio and could be maintained for more than 6 months.

DISCUSSION

Melanocytes in the vitiligo patches are destroyed by various mechanisms. Several hypotheses that have been postulated for the pathogenesis of vitiligo theorize the destruction of melanocytes by one mechanism or another (Lerner, 1971; Naughton et al. 1983; Puri et al. 1987). With the advent of techniques for growing melanocytes in culture (Eisinger and Marko, 1982; Gilchrest et al. 1984; Puri et al. 1987; Pittelkow and Shipley, 1989; Gordon et al. 1989; Medrano and Nordlund, 1990), research for elucidating the mechanisms underlying pigmented disorders became active.

Many different conditions of media in optimal melanocyte culture have been reported. PMA promotes melanocyte proliferation and inhibits keratinocyte growth at optimal concentration. Cholera toxin which was not used in our experiments is a potent inducer of c-AMP which results in growth inhibition of fibroblasts but is not a growth promoter of melanocytes (Pittelkow and Shipley, 1989). Basic fibroblast growth factor (bFGF) is the natural growth factor for human melanocytes (Halaban et al. 1987). BPE contains numerous large and small molecular weight polypeptides and other biological substances and is known to contain several mitogenic agents for mammalian cells. BPE contains a potent mitogen for melanocyte proliferation in vitro (Pittelkow and Shipley, 1989).

Now MCDB-153 media are widely used in the culture of melanocytes. Using this system, melanocytes could be successfully cultured from both foreskin and arm skin. Arm skin melanocytes could be easily obtained from suction blister roofs. Usually the epidermal roofs of suction blisters contain melanocytes and keratinocytes but not fibroblasts, since blister formation occurs at the dermoepidermal junction (Kistala and Mustalchillio, 1967). Tomita et al. (1985) reported that they have repeatedly obtained pure melanocytes from the epidermal roofs of suction blisters, and the yield of pure melanocytes by their method was far greater than by other methods. Culture of melanocytes using suction blistered epidermal sheets made it possible to obtain melanocytes from arm skins of many normal subjects and vitiligo patients without using dermatomes and anesthetics. This technique can be easily adapted to grow melanocytes or keratinocytes from individuals from different subjects and applied to patients with diseases of melanocyte or keratinocyte dysfunction. This will be a practical method which can be implemented in many different types of investigation in dermatology.

Puri et al. (1987) observed that melanocytes from uninvolved skin of vitiligo subjects manifested a lag of 8-11 days for the onset of growth and a decreased seeding capacity and that melanocytes could not be passaged. Puri et al. (1989) also reported that growth defects of melanocytes from vitiligo patients could be spontaneously corrected in repigmenting subjects and partially corrected by the addition of fibroblast-derived growth factors in vitro. Based on the above findings, they suggested a new hypothesis for the etiology of vitiligo that the melanocytes of vitiligo patients had inherent cellular defects. In 1990, Medrano and Nordlund reported that adult and vitiligo melanocytes could be cultured with modified current standard culture conditions. They concluded that in vitro long-term growth of human adult melanocytes from normal and vitiligo donors appeared to be dependent on the external growth factors and antioxidants. They also reported that little or no growth differences were observed between vitiligo and normal melanocytes. Our study also revealed that normal and vitiligo melanocytes showed no differences in growth characteristics and morphology.

And these findings are consistent with other histopathologic approaches. Moellman et al. (1982) found vacuolated degenerating keratinocytes in the perilesional and uninvolved skin of vitiligo patients, while the melanocytes were apparently normal. In contrast, recently published data suggested that vitiligo melanocytes contained an inherent cellular de-
fect. Boissy et al. (1991) reported that cultured melanocytes from vitiligo patients demonstrated abnormalities of structural aberration of the rough endoplasmic reticulum and melanosome compartmentalization in their ultrastructural study. They also demonstrated that melanocytes from most vitiligo patients expressed an innate defect when cultured and this abnormality might be the primary defect that elicited melanocyte destruction in vivo. The inherent melanocyte defect theory in the pathogenesis in vitiligo is now in heated debate. Melanocytes of vitiligo donors can be cultured with appropriate culture systems and no differences are found in our study, but further functional studies on tyrosinase activity assay, melanin content, thymidine uptake tests, and responses to external stimuli such as ultraviolet radiation or various cytokines and detailed morphological studies on the ultrastructural characteristics of melanocytes will be necessary to clarify the pathogenesis of vitiligo.

REFERENCES


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