In Vitro Growth Characteristics of Melanocytes Obtained From Adult Normal and Vitiligo Subjects

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The in vitro growth characteristics of melanocytes obtained from uninvolved and perilesional skin of vitiligo vulgaris subjects have been investigated in comparison to those from healthy adult donors. Normal human melanocytes have been found to grow exponentially in the presence of 10^{-11} M cholera toxin and 10 ng/ml of 12-*O*-tetradecanoylphorbol-13-acetate in routine tissue culture media. They could be trypsinized up to 3–4 passages. Melanocytes of the uninvolved skin of vitiligo subjects manifested a lag of 8–11 days for the onset of growth and they could not be passaged. Melanocytes obtained from both hypo- and hyperpigmented perilesional skin failed to grow under these conditions. Only in a few cases where the perilesional skin was normally pigmented did the melanocytes manifest some growth after a lag of 15 days. The initial seeding capacity of the melanocytes from uninvolved and perilesional skin of vitiligo patients were, respectively, 50% and 25% of the normal individuals. Vitiligo lesions themselves gave rise to unidentified dendritic cells that survived for 10–15 days without manifesting any growth. Our results suggest that melanocytes of individuals with vitiligo are defective. This fact has to be taken into account in any theory on the etiology of vitiligo. *J Invest Dermatol 88:434–438, 1987*

Although vitiligo affects approximately 1% of the population, its etiology is far from clear [1]. Current theories suggest that melanocytes in the vitiligo patches are destroyed by various mechanisms [2–4]. However, tyrosinase, the marker enzyme for melanocytes, has been detected in vitiligo lesions, suggesting the existence of inactive melanocytes therein [5]. To date no attempts to culture melanocytes from vitiligo subjects have been made.

We therefore thought that investigation on the dynamics of growth of melanocytes from vitiligo subjects would be important in understanding the etiology of this disease. In this communication we present data to demonstrate that melanocytes from perilesional and uninvolved areas of vitiligo patients manifest defective growth and passage capacities. Based on these findings, we conclude that a defect(s) in melanocytes may be important in the etiology of vitiligo, in addition to the degeneration of keratinocytes demonstrated by Nordlund and coworkers [6].

MATERIALS AND METHODS

Eagle's minimum essential medium, the nonessential amino acids, fetal calf serum, and antibiotics were from GIBCO, Grand Island, New York. Cholera toxin (CT), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and trypsin were purchased from Sigma, St. Louis, Missouri, Consolidated Midland Corporation, Katonah, New York, and Difco, U.S.A., respectively. Other chemicals used were of the highest grade available locally.

Baby foreskins were obtained from Lady Harding Medical College, New Delhi, India. Normal adult skin was taken from donors undergoing plastic and cosmetic surgery at our hospital. Vitiligo patients appearing at our hospital Pigmentation Clinic and at the Vitiligo Clinic run by the Central Council for Research In Unani Medicine at Tibbia College, New Delhi, were screened carefully. Only patients with common vitiligo (vitiligo vulgaris) who had not taken any medicine for at least 1 year were selected for the study. After informed consent was obtained, intermediate-thickness split skin biopsies were obtained under local anesthesia (1% lidocaine) from 3 different areas: vitiligo affected, perilesional (pigmented-vitiligo interface), and uninvolved normal skin. The details of the biopsies and case histories of the patients investigated are presented in Table I.

Culture Techniques

Baby Foreskins: The processing of baby foreskins and culture conditions were essentially similar to those described by Eisinger and Marko [7]. The epidermal cell suspensions were plated in 25-cm^2 Falcon flasks at a density of 2 million cells/flask, in the presence of CT and TPA.

Adult Skins: The epidermal cell suspensions prepared by overnight trypsinization were seeded in Linbro (Flow Laboratories, McLean, Virginia) 24-well plates in the absence of CT at densities of 0.25 million cells/well in the case of normal individuals and 0.75–1.0 million cells/well in the case of vitiligo individuals. These minor modifications in the technique of Eisinger and Marko [7] had to be made because preliminary studies indicated that vitiligo subjects' skin yielded fewer melanocytes. The floating cells were aspirated and fresh media containing 10 ng/ml of TPA and required amount of CT were added 48 h after the initial seeding. The media were replaced every 3 days.

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Abbreviations:
CT: cholera toxin
ISC: initial seeding capacity
TPA: 12-*O*-tetradecanoylphorbol-13-acetate

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Table I. Case Histories of Vitiligo Subjects Investigated in the Present Study

Case No.	Age (years)	Sex	Duration of Disease (years)	Percent Body Involved	Clinical Course[a]	Site of Biopsy		
						Uninvolved	Perilesional	Affected
1	22	M	8	10	P.N.P.	Thigh	Thigh	Thigh
2	28	F	6	10	R.P.N.P.	Trunk	Trunk	Trunk
3	47	F	6	35	P.N.P.	Thigh	Thigh	Thigh
4	25	F	16	70	P.C.N.P.	Thigh	Thigh	Thigh
5	38	F	20	30	S.	Arm	Thigh	Thigh
6	38	M	22	35	S.	Thigh	Thigh	Thigh
7	23	F	9	15	P.N.P.	Thigh	Thigh	Thigh
8	18	M	6	10	S.	Thigh	Thigh	Thigh
9	24	M	7	95	P.N.P.	—	Arm	Arm
10	25	F	20	40	P.N.P.	—	Trunk	Trunk
11	32	M	10	30	S.	Thigh	Trunk	Trunk
12	45	M	30	50	P.N.P.	Trunk	Trunk	—

Cases 6, 7, and 10 had normally pigmented perilesional skin. Cases 8 and 11 had hyperpigmented borders, while others had hypopigmented borders.
[a]P.N.P. = progressing with new patches appearing
R.P.N.P. = rapidly progressing with new patches appearing
P.C.N.P. = progressing centrifugally as well as new patches appearing
S = stable

Cell Counting: Adult melanocytes with their dendrites were easily identified and in situ counts of at least 10 randomly selected microscopic fields (0.49 mm^2) were taken according to the method outlined by Aubock et al [8].

Cell Passages: Differential trypsinization was carried out as outlined by Eisinger and Marko [7]. The baby foreskin melanocytes at near confluency were trypsinized and plated at the split ratio of 1:2. Adult melanocytes originating from both vitiligo and normal subjects were trypsinized when they reached population densities of more than 90 per field and plated into single wells. Fibroblasts, if present, were identified under phase microscopy [9] and, in later passages of baby foreskin melanocyte cultures, were removed by geneticin treatment [10].

RESULTS

Baby Foreskin Melanocytes The purpose of establishing baby foreskin melanocytes in culture was to reproduce the results of earlier workers under our laboratory conditions. As can be seen from Fig 1, the morphology and behavior of baby foreskin melanocytes in culture were essentially similar to those found by Eisinger and Marko [7]. The baby foreskin melanocytes are at present between 10–14 passages and are growing well. Fibroblasts, when present, could be eliminated by geneticin treatment.

Adult Skin Melanocytes Unlike baby foreskin melanocytes, adult melanocytes did not grow in the presence of 10^{-8} M CT (Fig 2). The ideal concentration of CT was found to be 10^{-11} M for these melanocytes (Fig 3). Under these culture conditions, there were no morphologic differences between normal and vitiligo melanocytes in the initial stages of primary cultures (Fig 1); however, there were differences in their growth patterns (Fig 3). Melanocytes obtained from normal individuals showed linear growth characteristics irrespective of the age of the donor. In contrast, melanocytes obtained from normal regions of vitiligo subjects exhibited a lag of 8–11 days for the onset of growth phase, while melanocytes originating from hypo- and hyperpigmented borders failed to grow under these culture conditions. Only in those cases where the border area was normally pigmented (Cases 6 and 7) did the melanocytes exhibit some growth after a lag of 15 days (Fig 4—only results of Case 6 are shown). Once the lag was over, the melanocytes of normal-appearing areas of vitiligo subjects grew at rates comparable to those of normal subjects. These differences in the growth characteristics were not due to low melanocyte seeding densities in the case of vitiligo subjects (see below), as melanocytes of the normal subjects which were seeded at densities similar to those of vitiligo subjects manifested no lag before the onset of growth phase (Fig 3).

Initial Seeding Capacity (ISC) This is defined as the number of melanocytes per microscopic field (0.49 mm^2) 48 h after seeding 10^6 epidermal cells as described above. An average ISC of 123.84 ± 14.52 was found in the present study for normal control individuals varying in age from 19 to 62 years (Table II). In vitiligo subjects, ISC has been found to be 52.31 ± 11.06 for contralateral normal skins and 26.47 ± 3.69 for adjacent border skins (Table II).

Passages The melanocytes obtained from normal healthy subjects could be trypsinized and passaged up to 3–4 times. In contrast, melanocytes originating from vitiligo subjects failed to grow after trypsinization in most cases. Only in 1 case (7) could the contralateral normal skin melanocytes be passaged to 2 passages. The reason for this exception remains unclear. However, it may be pointed out that Case 7 had normally pigmented perilesional skin, was relatively younger, and the duration of the disease was 9 years (Table I). Microscopically, the melanocytes of vitiligo subjects that seeded into new wells after trypsinization in most cases initially appeared normal, but within a few days became hyaline and within a month were all dead.

Vitiligo Skin The vitiligo-affected patches gave rise to a few dendritic cells that survived more than 10 days under culture conditions designed for the selective proliferation of melanocytes. Only in 1 case did their numbers per field increase. An observation that may be of some significance is that while an occasional fibroblast contamination was seen in normal skin, uninvolved, and perilesional area-derived melanocyte cultures, not a single vitiligo lesion investigated so far gave rise to this cell.

DISCUSSION

Human skin melanocytes have now been established to grow in routine tissue culture medium supplemented with 10 ng/ml of TPA and 10^{-8} M CT [7,11], as well as in chemically defined media supplemented with a hypothalamic-derived factor and 10^{-9} M CT [9]. Under these conditions, baby foreskin melanocytes have been found to grow for more than 30 passages and those derived from healthy adult skin for 3–4 passages. The results of the present study are in agreement with these findings, except that in our study we found that adult melanocytes grow well in 10^{-11} M CT (Fig 3). The discrepancy in the requirement of different levels of CT in these studies for the optimum growth of adult melanocytes may be due to the differences in the age and intrinsic melanogenic levels of the donors.

It is generally agreed that melanocytes are in normal numbers in the uninvolved skin of vitiligo patients [1,12]. In the present study we have found that the initial melanocyte seeding capacity

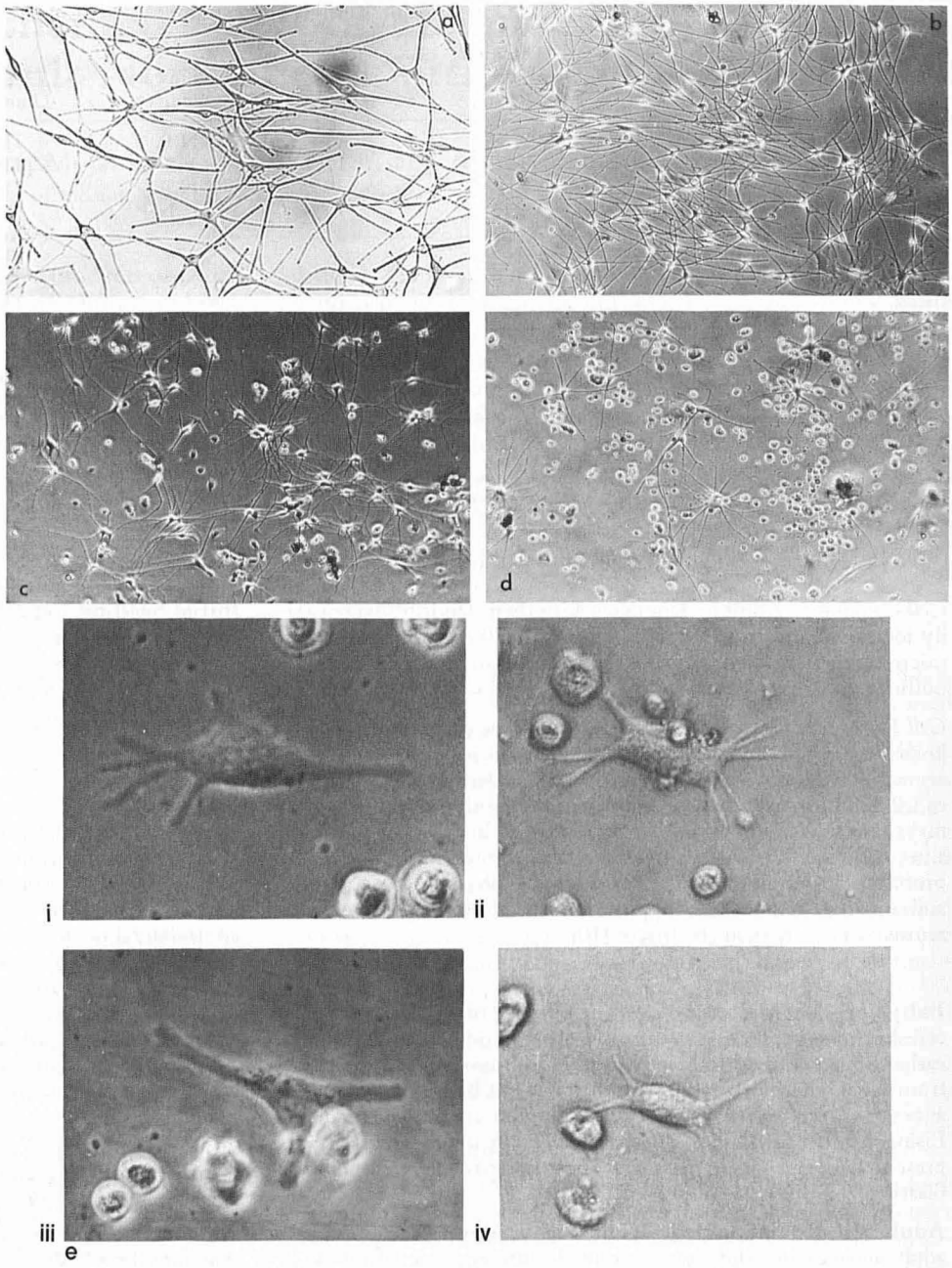

Figure 1. Melanocytes of normal and vitiligo subjects. Melanocytes with their prominent nuclei and long thin dendrites can be easily distinguished from the other skin cells. Morphologically, melanocytes obtained from vitiligo subjects were similar to those from normal donors. *a,* Baby foreskin melanocytes, fourth passage. × 150. *b,* Normal adult human skin melanocytes (Table II, Case 15), first passage. Phase contrast, × 92. *c,* Melanocytes from uninvolved skin of vitiligo subject (Table I, Case 8), 18th day of primary culture. Unattached keratinocytes can also be seen. Phase contrast, × 92. *d,* Perilesional melanocytes from vitiligo subject (Table II, Case 11), 24th day of primary culture. Phase contrast, × 92. *e,* Unidentified dendritic cells seen in cultures from vitiligo lesions: (*i*) Case 8 (Table II), 5th day; (*ii*) Case 11 (Table II), 7th day; (*iii*) Case 5 (Table II), 6th day; (*iv*) Case 9 (Table I) 7th day of culture. × 370.

of the normally pigmented skin of vitiligo patients is reduced to 50% of healthy human skin (Table II). In the clinically hypopigmented borders, melanocytes are believed to be either normal or reduced in number [1]. The initial melanocyte seeding capacity of the skin adjacent to the affected areas is found to be 25% of the normal individuals (Table II). This reduced melanocyte seeding capacity suggests that despite their being normal in number and morphologic appearance, the melanocytes from vitiligo patients are different from those of normal individuals. The initial seeding capacity estimated in the present study for normal donors is comparable to the melanocyte yield calculated by Gilchrest et al [9].

The low melanocyte yield (Table II) and the lag before the onset of growth (Fig 3) of the normally pigmented skin melanocytes of vitiligo subjects could be interpreted to be due to the time required for the melanocytes to recover from the effects of circulating melanocyte antibodies [3], lymphokines, and lyso-

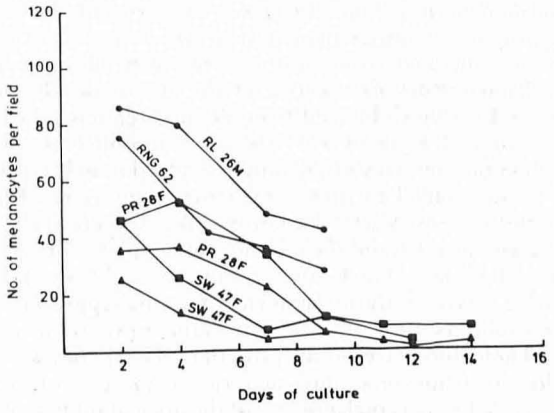

Figure 2. Melanocytes from normal (Cases 13 and 17, Table II) and uninvolved skin of vitiligo patients (Cases 2 and 3) grown in 10^{-8} M CT.

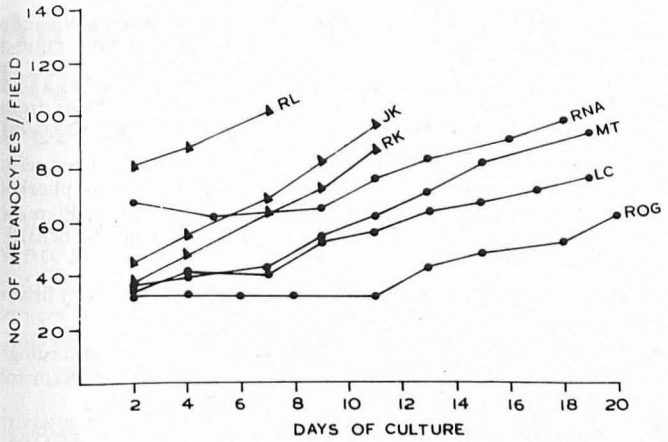

Figure 3. Melanocytes from normal donors grow in the presence of 10^{-11} M CT without manifesting any lag. Those from uninvolved skin manifest a lag of 8–11 days for growth. Melanocytes from normal donors seeded at lower densities (Cases 14 and 16, Table II) do not manifest lag for the onset of growth. *Triangles* = normal donors; *circles* = uninvolved skin of vitiligo subjects. See text for more details.

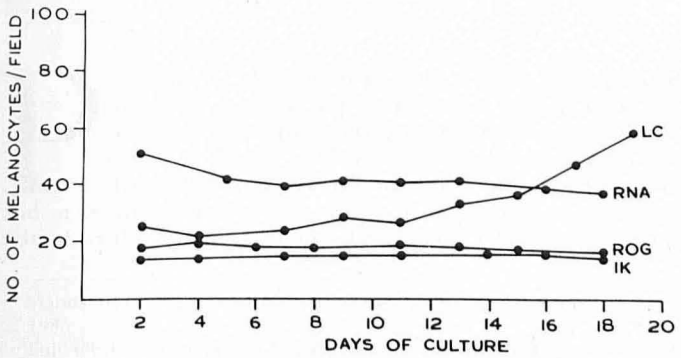

Figure 4. Melanocytes except those obtained from normally pigmented perilesional skin (Case 6, Table I) do not manifest any growth. Culture conditions are described in the text.

somal enzymes from Langerhans cells, etc. [13]. Nordlund and colleagues found vacuolated degenerating keratinocytes in the perilesional and uninvolved skin of vitiligo patients, while the melanocytes were apparently normal [6]. It is therefore also possible that the keratinocytes persisting unattached or partially attached in the primary cultures might elaborate some substance that contributes to the growth lag of the melanocytes from the uninvolved skin of the vitiligo subjects. However, these melanocytes in their post-lag period grow at rates comparable to those obtained from normal donors, despite the presence of unattached keratinocytes (Fig 3). The lack of growth of these melanocytes after trypsinization of the growing primary cultures from vitiligo subjects indicates that the primary defect in this disease could be intrinsic to the melanocytes themselves.

Trypsinization is known to remove many cell surface proteins [14] and normal human melanocytes are highly sensitive to this enzyme [11]. Melanocytes from vitiligo subjects appear to be even more sensitive to this enzyme as revealed by their low melanocyte yield following trypsinization of the skin to get epidermal cell suspensions (Table II) and their inability to be passaged. Preliminary findings in our laboratory have indicated that melanocytes from vitiligo subjects can be successfully passaged after trypsinization in the presence of fetal lung fibroblast supernatants suspected to contain melanocyte growth factors [15]. These results suggest that the basic defect in vitiligo could be the inability of these melanocytes to respond to levels of growth stimuli that are enough to stimulate growth in normal human melanocytes either because of the presence of specific inhibitors of growth factor(s) or because of a decreased number of surface receptors on vitiligo melanocytes for the growth factors.

Previous work from our laboratory has revealed that vitiligo lesions contain 4–32% of the tyrosinase activity of normal adjacent human skin [5]. In the present study we have found that vitiligo skin gives rise to a few dendritic cells in culture that survive for 10 or more days under conditions optimum for melanocyte proliferation. Under these conditions the Langerhans cells are known to survive only 3–4 days [7]. Whether the dendritic cells observed in our study are α-dendritic cells/effete melanocytes as observed by Mishima et al [16] and require larger doses of proliferation and melanogenic factors to induce growth and melanogenesis or some other type of dendritic cells remains to be elucidated.

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Table II. Initial Seeding Capacity of Melanocytes Derived From Normal and Vitiligo Subjects

Case No.	Age (years)	Sex	Area of Biopsy	Initial Seeding Capacity		
				Normal Subject	Vitiligo Subject	
					Uninvolved	Perilesional
13	62	M	Thigh	101.20	—	—
14	32	F	Thigh	125.33	—	—
15	24	F	Forearm	136.50	—	—
16	19	M	Thigh	115.00	—	—
17	26	M	Thigh	141.20	—	—
5	38	F	Arm/thigh	—	68.12	28.80
3	47	F	Thigh/thigh	—	52.50	25.60
4	25	F	Thigh/thigh	—	44.80	24.40
2	28	F	Trunk/trunk	—	50.20	30.00
8	18	M	Thigh/thigh	—	67.50	29.30
11	32	M	Thigh/trunk	—	48.00	28.50
12	45	M	Trunk/trunk	—	35.10	18.70
		Mean ± SE		123.84 ± 14.52	52.31 ± 11.06	26.47 ± 3.69

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