Melanocytes and Langerhans Cells in Aged Versus Young Skin Before and After Transplantation onto Nude Mice

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Previous studies have demonstrated decreased numbers of melanocytes and Langerhans cells (LC) in aged skin. In the present study, we employed dopa and indirect immunoperoxidase techniques in epidermal sheets to determine the fate of melanocytes and LC of aged versus young donors after skin transplantations onto nude mice. The detection of positive homologous leucocytic antibody reaction of degeneration (HLA-DR) of LC indicates an age-associated reduction in sun-protected thigh skin in aged versus young subjects (263 ± 63 versus 589.25 ± 142.643, $p < 0.001$). The mean number of LC four weeks after transplanation remained almost constant. Prior to skin engraftment, a decreased number of melanocytes was found in aged versus young epidermis (160.77 ± 51.7 versus 255.83 ± 81.2, respectively, $p < 0.05$). A significantly increased number of melanocytes was noted four weeks following engraftment in epidermis from aged (307.44 ± 174, $p < 0.05$) and young human donors (402.16 ± 139, $p < 0.02$). The marked increase in density of dopa-positive melanocytes following engraftment onto nude mice may indicate the existence of circulating factors in nude mice that perhaps both stimulates and enhances proliferation and activity of these cells. *J Invest Dermatol 96:210–214, 1991*

Observations by Krueger et al [1] and our unpublished observations have demonstrated striking pigmentation in human split-thickness skin grafts (HSTSG) four to six weeks after transplantation onto nude mice, but not onto nude rats [2]. Faint pigmentation has been noted in HSTSG transplanted onto skin of dark colored subjects. Maximal pigmentation was noted one year after transplantation [3], and decreased numbers of melanocytes were found in the hyperpigmented group [3,4].

In the present study, we employed dopa staining in epidermal sheets before and after engraftment of HSTSG onto nude mice, to determine the fate of dopa-positive melanocytes. Increasing numbers of these cells may explain the striking pigmentation of the grafts. In addition, we used this model to study the pigmentary capacity of aged, unexposed skin. A decreased number of melanocytes in aged skin has been well documented [5–7]. However, in aged and young epidermis the number of melanocytes is less in the unexposed areas compared to the exposed ones [6,8]. It has been shown that ultraviolet radiation (UVR) increases the dopa-positive melanocytes of unexposed skin [6,9]. The aim of the present study was to determine whether aged, unexposed skin has the potential ability to become pigmented compared to young, unexposed skin. Moreover, we thought that it would be of interest to compare the number of dopa-positive melanocytes in the epidermis of the aged to that of the young before and after skin transplantation. Furthermore, we felt that it would be of interest to determine the number of HLA-DR–positive Langerhans cells (LC) in the same HSTSG because of the relatively low incidence of allergic contact dermatitis in the older population [10]. Gilchrest et al [11] have previously found that the number of LC in the epidermis of cross-section specimens of human skin decreases by almost 50% between early and late adulthood. This reduction may be a factor in the age-associated decrease in immune responsiveness observed in the skin [12].

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Abbreviations:
HSTSG: human split-thickness skin graft
LC: Langerhans cells
PBS: phosphate-buffered saline
STSG: split-thickness skin graft
UVR: ultraviolet radiation

MATERIALS AND METHODS

Patients Nine volunteers, age 73 to 89 (mean age 82.3), known to be in good health, and nine young, healthy medical students, age 23 to 28 (mean age 24.5) were recruited for this study after giving informed consent. All subjects were classified as skin type I–IV according to Fitzpatrick's criteria [13]. From each subject, 3-mm diameter punch biopsies and 0.4-mm split-thickness skin grafts (HSTSG) were obtained using a dermatome knife. Punch biopsies were obtained for the dopa procedure (nine donors in the group of older persons, and six in the group of younger persons) for detection of the number of LC by using monoclonal antibodies specific for HLA-DR (eight donors in each group) and routine histology, whereas the STSG were grafted onto nude mice. The donor grafts were approximately 1.2–1.5 cm in diameter. The STSG and the biopsies were taken from the thighs after local anesthesia with 1% lidocaine.

Animals The animals used in this study were 2- to 3-month old outbred Balb/C nude mice, obtained from the pathogen-free animal breeding facility at the University of Tel Aviv, and raised in the pathogen-free animal facility of the Faculty of Medicine, Technion-Israel Institute of Technology, Haifa.

Skin Grafting The split-thickness skin graft was transplanted onto the subcutaneous tissue over the lateral thoracic cage of the

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Table I. The Number of Positive Dopa-Positive Melanocytes/mm² in Aged and Young Skin Before and After Transplantation onto Nude Mice

	Aged Skin				Young Skin			
Subject	Age	Skin Type	Before Grafting	After Grafting	Age	Skin Type	Before Grafting	After Grafting
1	73	III	135 ± 10.8	204 ± 13.4	24	III	225 ± 13.5	348 ± 12.2
2	76	III	141 ± 13.8	321 ± 15.4	24	III	278 ± 14.6	546 ± 15.8
3	77	II	283 ± 12.8	253 ± 19.3	23	III	225 ± 10.3	510 ± 19.3
4	84	III	175 ± 13.7	124 ± 10.4	24	II	162 ± 8.9	207 ± 13.85
5	84	II	169 ± 7.4	203 ± 20.3	28	II	242 ± 12.1	291 ± 14.7
6	83	III	95 ± 5.6	137 ± 7.4	24	III	403 ± 15.0	511 ± 20.1
7	86	III	161 ± 12.1	431 ± 17.5				
8	89	II	133 ± 12.7	449 ± 20.5				
9	89	III	155 ± 10.9	655 ± 19.8				
Mean ± SD[a]			160.77 ± 51.7	307 ± 174.4			255.83 ± 81.2	402.16 ± 139.7

[a] Difference between the mean number of melanocytes per mm² in aged and young epidermis before skin transplantation ($p < 0.05$) and after skin transplantation (N.S.). A statistically significant increase in the number of cells was noted in the post-transplantation epidermis in both young ($p < 0.05$) and aged ($p < 0.02$) skin.

mouse, as previously described [3]. Graft sites were covered with petroleum-impregnated gauze and standard adhesive bandages, surgically stapled to the ventral surface of the animals.

Quantitative Analysis of Dopa-Positive Melanocytes Skin biopsies were evaluated for melanocyte density prior to and four weeks after skin transplantation as previously described [14]. The epidermis was separated by immersing the skin in 2 N sodium bromide for 45 min at 37°C. Epidermal sheets were subsequently incubated with 0.1% dopa (L-3,4-dehydroxyphenylanine) phosphate-buffered saline (pH 7.4) for 3 to 5 h at 37° C. Following incubation, the epidermal pieces were then fixed in a 10% formal saline solution, mounted on glass slides with the dermo-epidermal junction facing upward, and then examined and photographed using a Photomicroscope III Zeiss.

Epidermal melanocytes were counted in each specimen using an ocular grid in 10 independent fields at ×400 magnification. The average number of dopa-positive melanocytes was estimated per square millimeter of surface area. Biopsies for routine light microscopic examination were fixed in 10% formaldehyde embedded in paraffin. Sections from each specimen were stained with hematoxylin-eosin.

Quantiative Analysis of LC LC density of the human epidermal sheets was quantitated by using monoclonal antibodies specific for HLA-DR. Monoclonal antibodies specific for HLA-DR antigens that were used in this study include antiHLA-DR, a mouse monoclonal antibody against human DR antigens, which binds to the Ia-like antigens of human LC (Becton-Dickinson, Palo Alto, California) and HLA-ABC (Code, MAS 114P; clone, BI-303, Sera-Lab, Sussex, England), a mouse monoclonal antibody that was used to distinguish human from mouse tissue.

Before and after skin transplantation, the skin grafts were removed from the mice and placed in a solution of ethylenediaminetetra-acetic acid (EDTA) as described previously [15]. After incubation for 2 h at 37°C, the epidermal sheet was carefully peeled from the dermis and cut into four pieces. Thereafter the pieces of the epidermal sheet were fixed in cold acetone, rehydrated with phosphate-buffered saline (PBS), incubated for 1 h with the test antibody, and washed with PBS. The presence of tissue-bound antibody to target antigen was assessed by the indirect immunoperoxidase technique (Vectastain ABC immunoperoxidase-staining procedure and reagents, mouse IgG PK 4002 kit, Vector Laboratories Incorporated, Burlingame, CA). Incubation with the secondary reagents was for 30 min at room temperature, followed by washing with PBS and incubation for 30 min with ABC reagent. After washing with PBS the sections were incubated for 5 to 7 min in 3-amino-α-ethylcarbazol. Sections were washed in tap water before being mounted in glycerol and examined with a Zeiss microscope. The density of the cells was determined by random counts in 15 fields at ×400 magnification using an ocular grid of known area. Results were expressed as number of LC/mm².

Statistical analysis was carried out using the Student t test.

RESULTS

General Observation One week after transplantation all grafts were pink in color. Four weeks following engraftment, all groups showed marked hyperpigmentation, except for the graft from subject number 4, which remained pink four weeks following engraftment. Longer follow-up revealed pigmentation of this graft during the 5–6 weeks of transplantation.

Histologic Findings Table I and Fig 1 demonstrate the mean number of melanocytes before and after skin transplantation. Prior to skin engraftment, the mean number of melanocytes was found to be significantly lower in aged epidermis (Fig 2) (160.77 ± 51.7 versus 255.83 ± 81.2, $p < 0.05$). A significantly increased number of melanocytes was observed four weeks following engraftment in epidermis from aged (307.44 ± 174, $p < 0.05$) and young human donors (402.1 ± 139, $p < 0.02$) (Fig 3). There was, however, a small but insignificant difference between the two groups. Table I demonstrates the number of melanocytes in each graft before and after engraftment. An increasing number of melanocytes was observed in seven out of nine grafts obtained from aged human donors, whereas all six grafts obtained from the young donors showed an increased number of cells. Grafts obtained from subjects 3 and 4 of

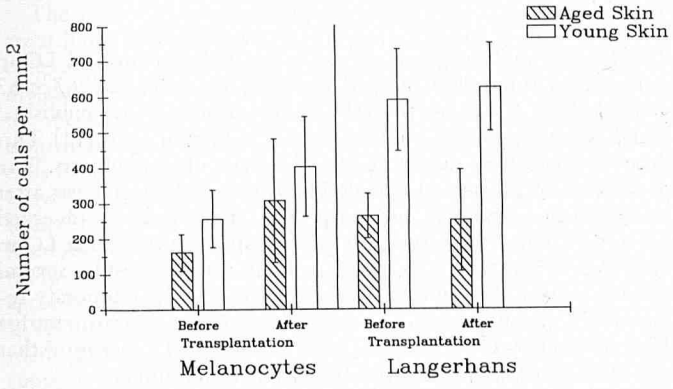

Figure 1. Analysis of mean number of melanocytes and Langerhans cells per mm² (±SD) before and after skin grafting. More cells were observed in aged versus young skin before grafting. One month after grafting the mean number of LC remained almost constant, whereas a significant increase in number of melanocytes were noted.

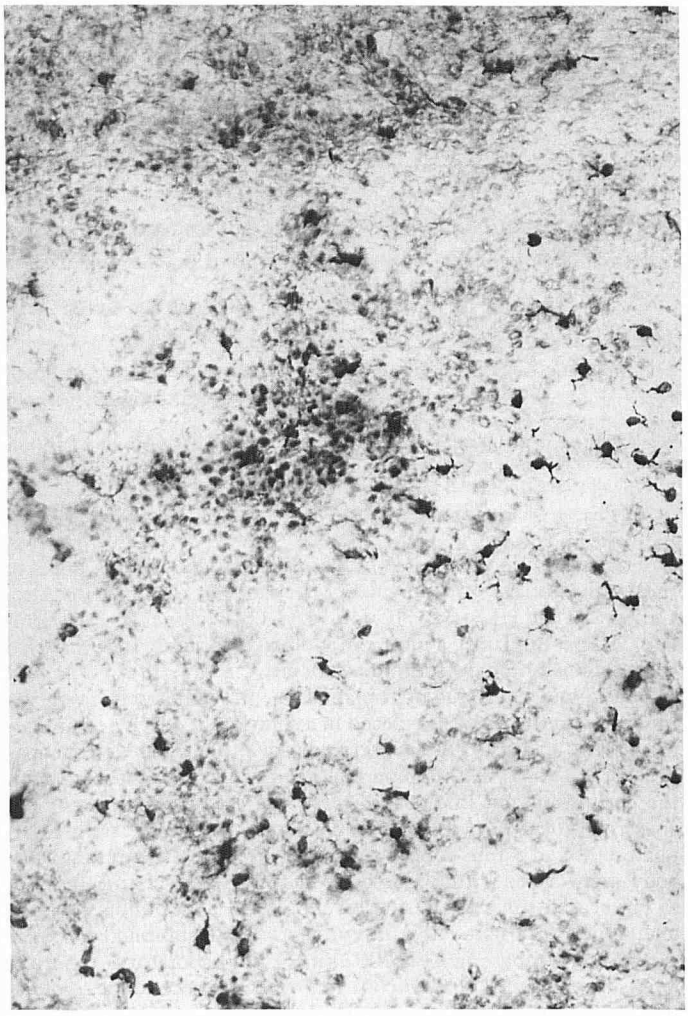

Figure 2. Dopa-positive melanocytes in the epidermal sheet of aged skin before grafting (×300).

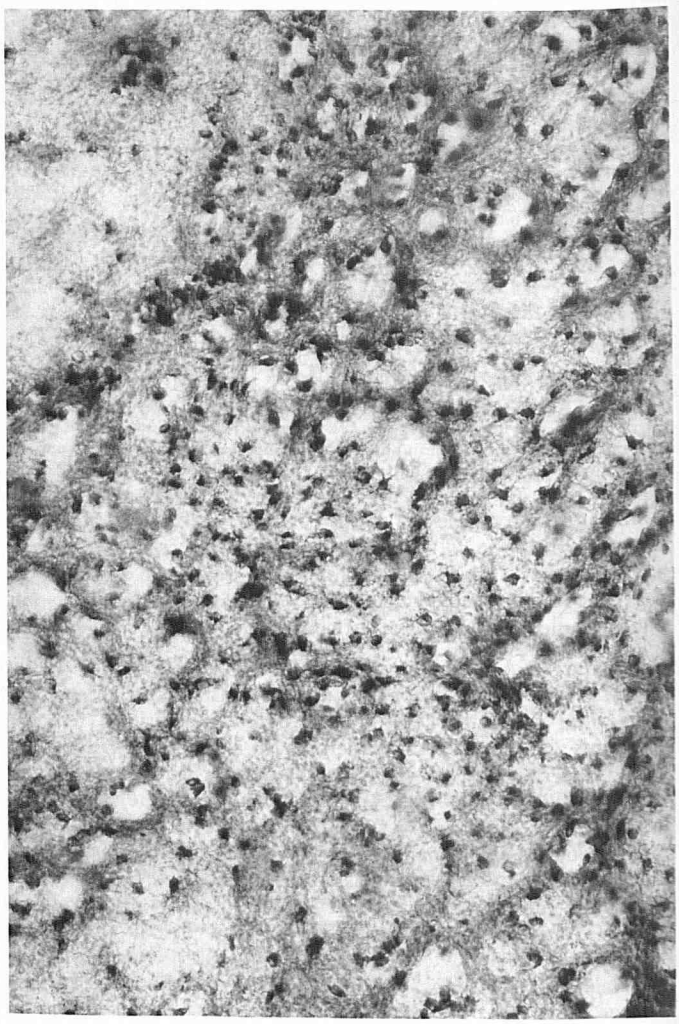

Figure 3. Dopa-positive melanocytes in the epidermal sheet of aged skin after grafting. Note the increasing number of melanocytes after grafting versus before grafting shown in Fig 1 (×300).

the aged group showed a decreased number of melanocytes. After grafting, dopa positivity or darker staining for melanocytes was noted in all specimens. In subjects 2, 6, 7, and 8 the number of melanocytes was also detected 6 and 12 weeks after transplantation (data not shown in Table I). A similar number of cells was found 4, 6, and 12 weeks after transplantation. Thus, the increased number of these cells was noted mostly after the first month of transplantation. The dendrites of melanocytes became thicker and more expansive, and were noted mostly 12 weeks after transplantation. Routine light microscopic examination of unstained frozen sections revealed prominent deposition of melanin granules in the epidermis and the upper dermis four weeks after transplantation. This feature was noted in most grafts only after skin transplantation.

Table II and Fig 1 demonstrate the mean number of LC in aged versus young epidermis before and after engraftment of HSTSG onto nude mice. The number of LC, four weeks after transplantation, remained almost constant. The mean number of aged epidermis was 250.25 ± 46.232 versus 565.62 ± 123.44 ($p < 0.001$) in young skin. Table II demonstrates the number of LC in each subject. No striking difference was noted before and after skin transplantation. The number of LC was also determined 6 and 12 weeks after transplantation of skin obtained from subjects 2 and 8 of the aged group (data not shown in Table II). The number of cells remained nearly constant in skin obtained from subject 2 (day 0, 286; day 30, 278; day 44, 298; day 88, 278). Reduction of cells was observed in skin obtained from subject 8, four weeks after transplantation. However, no striking changes were observed thereafter (day 0, 274; day 30, 207; day 44, 200; day 88, 215).

DISCUSSION

The present study suggests an age-associated reduction of LC in unprotected thigh skin in aged versus young subjects (263 ± 63 versus 589.25 ± 142.64, $p < 0.001$). Gilchrest et al have published a study showing similar, but less striking, reduction in LC [11]. This difference could be related to the older age of our subjects. The persistence of positive HLA-DR LC in the epidermal sheets after skin transplantation is not surprising, having been observed previously [1,16]. However, the present study showed that LC in aged skin are also able to maintain their markers despite several factors, such as the trauma of transplantation and a temporary reduction of vascularity. Moreover, analysis of skin engraftment for 12 weeks (subject 2) revealed a mean number of LC per mm^2 that was almost identical to the pre-transplantation number.

The fate of melanocytes following engraftment of HSTSG onto nude mice had some similarity to the melanocyte response after UVR exposure. An increased number of these cells occurred on the buttocks of aged subjects after exposure to UVR [6,9]. Similarly, a

Table II. The Number of Positive HLA-DR LC/mm² in Aged and Young Skin Before and After Transplantation onto Nude Mice

	Aged Skin			Young Skin		
Subject	Age	Before Grafting	After Grafting	Age	Before Grafting	After Grafting
1	73	279 ± 33.1	257 ± 18.1	24	721 ± 29.3	623 ± 34.1
2	76	286 ± 20.3	278 ± 17.4	23	546 ± 17.6	528 ± 27.0
3	77	381 ± 20.7	329 ± 30.6	24	439 ± 15.7	400 ± 19.5
4	84	256 ± 14.0	247 ± 16.7	24	321 ± 20.1	360 ± 16.0
5	83	230 ± 16.3	251 ± 20.6	28	641 ± 31.3	634 ± 21.1
6	86	242 ± 19.3	260 ± 19.2			
7	89	156 ± 17.1	173 ± 13.3	24	673 ± 24.4	640 ± 35.3
8	89	274 ± 21.7	207 ± 13.4	25	692 ± 27.7	680 ± 36.3
9				23	681 ± 18.8	660 ± 20.9
Mean ± SD[a]		263 ± 63.08	250.25 ± 46.2		589.25 ± 142.6	565 ± 123.4

[a] Difference between the mean number of LC per mm² in aged and young epidermis before and after skin transplantation onto nude mice reached statistical significance ($p < 0.001$, by Student t test).

significantly increased number of melanocytes was noted in the present study, following engraftment of HSTSG obtained from the human thigh skin, onto nude mice. The increasing number of cells was observed in the aged and young epidermis. Moreover, a darker staining for melanocytes was noted in grafted skin similar to that noted in UVR-exposed areas [8]. Prior to HSTSG transplantation, a decreased number of melanocytes was found in aged versus young epidermis (160.77 ± 51.7 versus 255.83 ± 81.29, $p < 0.05$). After transplantation there was still a small but insignificant difference.

Cutaneous pigmentation is dependent on a number of processes, including the production and melanization of melanocytes and the transfer of melanosomes to keratinocytes and the pattern of aggregation of melanosomes within keratinocytes [17]. Thus, hyperpigmentation could be caused by an increased number of functional melanocytes, increased melanization of melanosomes, or a change in the size or distribution of melanosomes [17]. A slight hyperpigmentation is common in skin grafts after transplantation onto Black, Caucasian, or Asiatic subjects [3]. The hyperpigmentation reaches a peak at about the end of one year and then diminishes gradually. A decreased number of melanocytes was perceived in these hyperpigmented grafts [3]. Therefore, Mir and Mir [18] suggested that melanocytes are overactive secondary to altered cutaneous innervation. In our model, however, hyperpigmentation corresponded to an increased number of melanocytes. Increased numbers of melanocytes were observed in seven of nine HSTSG from aged donors versus six of six HSTSG obtained from young subjects. Sunburn and many other skin inflammatory processes are characterized by hyperpigmentation as a result of an increased number of melanocytes and an increase in melanin production by the melanocytes [19].

The precise factors regulating human epidermal pigmentation are still unknown [20]. The UVR may cause an increase of melanocytes owing to activation of pre-existing amelanotic melanocytes or melanocyte proliferation [9]. Previously it had been speculated that the increasing number of melanocytes is based only on the activation of these cells because melanocytes are highly differentiated cells of neural crest origin and therefore are incapable of division. However, Jimbow et al [21] showed mitotic activity of these cells in humans. Rosdahl and Szabo [22] showed that UVB radiation stimulated mitotic activity of melanocytes in mice. Thus, the increasing number of melanocytes of mice following UVB radiation should account for the increasing mitotic rate. Some reports show that UVR directly induces proliferation and melanin production in pigmented human melanocytes in culture [23,24]. Very little evidence supports the concept that the increasing number of melanocytes is due to activation of the cells [9]. We could not determine whether the dopa staining performed in the present study indicated that the increasing number of melanocytes following engraftment is caused by proliferation of cells, activation, or a combination of both events.

Previous studies using a cultured system have shown that a variety of tissue extracts [25] and basic fibroblast growth factor derived from keratinocytes [26] can stimulate melanocyte proliferation. Arachidonic acid and several of the cyclooxygenase products prostaglandin (PGE_2), prostaglandin $E_{2\alpha}$, and prostaglandin D_2 are increased following UVR [27]. Prostaglandin E_2, the major postanoid made by cultured keratinocytes [28], was found to stimulate proliferation of murine melanocytes in vivo [29]. Application of PGE_2 to murine skin stimulates both proliferation and melanization [30]. Recently, Morelli et al [31] found that among the arachidonic acid and its metabolites (eicosanoids) only leukotriene C_4 and leukotriene D_4 stimulated human melanocyte proliferation. Therefore, they concluded that these metabolites play an important role in post-inflammatory melanocyte proliferation. In a recent study, Gordon et al [32] demonstrate that keratinocytes in vitro release factors that significantly enhance the growth, melanin production, and dendricity of isolated melanocytes. Several known keratinocyte-derived factors, such as basic fibroblast growth factor, prostaglandin E_2, leukotriene B_4, and others, failed to stimulate melanocyte activity. Ultrafiltration experiments performed in this study showed that cultured keratinocytes released three diverse size fractions that influence cultured melanocytes. Collectively, these data demonstrated that keratinocyte-derived factors play a role in melanogenic activity. However, these data are based only on in vitro studies using a variety of culture systems. The increasing number of melanocytes in human skin grafted onto nude mice may suggest that nude mice serve as a unique in vivo model for studying the factors that influence melanocytic behavior. Transplantation of the skin onto the mice causes epidermal cells to release those factors that enhance melanocytes function. Alternatively, the existence of circulating factors in nude mice may stimulate and enhance the activity of melanocytes. Isolation of such factors may elicit a better understanding of melanocytic biology. Recently, we described the appearance of dopa-positive melanocytes in STSG obtained from patients with vitiligo and idiopathic guttate hypermelanosis [33] and increased numbers of these cells in patients with lichen planus following engraftment onto nude mice [14]. Thus nude mice may serve as a tool in the study of pigmentary disorders.

More sophisticated biochemical and histologic techniques should be employed before and after engraftment to distinguish between a change in the number of melanocytes and in tyrosinase activity.

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