STUDY

Treatment of "Stable" Vitiligo by Timedsurgery and Transplantation of Cultured Epidermal Autografts

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Objective: To optimize melanocyte/keratinocyte cocultivation and to evaluate the effectiveness of autologous cultured epidermal grafts in the surgical treatment of stable vitiligo.

Design: After optimization of melanocyte/keratinocyte cultures, achromic lesions were disepithelialized by means of programmed diathermosurgery (Timedsurgery) and covered with autologous epidermal grafts prepared from secondary cultures. Melanocyte content was evaluated by dopa reaction. The percentage of repigmentation was calculated using a semiautomatic image analysis system.

Setting: A biosafety level 3 cell culture facility and a dermatological department in a hospital.

Patients: Thirty-two patients carrying different types of vitiligo were admitted to the study and treated with autologous cultured epidermal grafts. Inclusion criteria were (1) failure of at least 2 standard medical approaches; (2) no therapy for at least 12 months; (3) absence of progression of old lesions, absence of appearance of new lesions, and absence of Koebner phenomenon within the past 18 months; and (4) absence of autoimmune disorders.

Results: One hundred five achromic lesions (a total of 6078.2 cm^2) were treated. The average percentage of repigmentation, evaluated after 12 to 36 months of follow-up, was 77%. Independent of the type of vitiligo, average percentages of repigmentation of extremities and periorificial sites were 8% (31.8 cm^2 repigmented/420.5 cm^2 transplanted) and 35% (17.6 cm^2 repigmented/50.0 cm^2 transplanted), respectively. Percentages of repigmentation of all other body sites ranged from 88% to 96% (4329.7 cm^2 repigmented/4675.2 cm^2 transplanted). Color matching was good and scar formation was not observed.

Conclusion: Cultured epidermal grafts can be considered a real therapeutic surgical alternative for "stable" but not lip-tip vitiligo.

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MELANOCYTES ARE neural crest–derived cells located mainly in the basal layer of the epidermis and in the matrix of hair follicles.[1] Melanocytes synthesize melanin pigment and transfer mature melanosomes to basal keratinocytes and are therefore responsible for skin color and protection against photocarcinogenesis.[1] Vitiligo is a common idiopathic skin disease[2-7] that affects 1% to 2% of the world's population, causes selective destruction of melanocytes, and leads to the development of achromic lesions.[7-11] The cosmetic disfigurement caused by vitiligo has profound psychological effects on patients and gives rise to serious emotional stress in approximately two thirds of them.[12,13] Psychosocial difficulties include depression, low self-esteem, social rejection, and even job discrimination.[12,13] Vitiligo is usually "active," ie, is characterized by progression of old lesions, development of new lesions, and appearance of white macules after trauma (Koebner phenomenon).[14] In patients affected by segmental vitiligo, the causative factor(s) usually disappears, leaving well-defined achromic lesions.[3] Similarly, generalized vitiligo can enter long phases of clinical quiescence[2-11,14] in which the size and number of lesions are stationary for several years and the Koebner phenomenon is absent.[14] This stage of the disease is therefore referred to as *stable* vitiligo. Several findings strongly support the view that melanocytes are eventually destroyed in stable vitiligo.[3]

Distinction between active and stable phases of the disease is important for selecting the more appropriate therapy. Active vitiligo usually requires medical therapy, which includes the use of psoralen, topical and oral administration of corticosteroids, khellin, or phenylalanine, along with UV-A radiation.[6-11,15,16] Surgi-

PATIENTS, MATERIALS, AND METHODS

INCLUSION CRITERIA

Patients were evaluated clinically, by a questionnaire, and by serological testing; patients presenting with autoimmune disorders (thyroid disease, diabetes mellitus, alopecia areata, pernicious anemia, and Addison disease) or with organ-specific circulating autoantibodies (antiparietal cells, antithyroglobulin, antithyroperoxidase, and antimicrosomal autoantibodies) were excluded from the study. Inclusion criteria were (1) failure of at least 2 standard medical approaches, (2) no therapy for at least 12 months, (3) lack of progression of old lesions within the past 18 months, (4) no new lesions developing within the past 18 months, and (5) absence of a Koebner phenomenon within the past 18 months.

Thirty-two patients (carrying 105 distinct achromic lesions) presenting with different types of vitiligo were enrolled in the study. The duration of clinical stability ranged from 1.5 to 50 years. Informed consent was obtained from all patients. Procedures followed were in accordance with the ethical standards of the Committees on Human Experimentation of our institutions.

CELL CULTURE

We cultured 3T3-J2 mouse cells in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum, glutamine (4 mmol/L), and penicillin-streptomycin (50 IU–50 µg/mL).

Full-thickess skin biopsy specimens (0.5-4 cm^2) were taken from unaffected and, when possible, hairy body areas. In particular, biopsy specimens were taken from the pubic area, buttocks, thighs, and abdomen. Keratinocytes were cultured as described previously,[28] with some modifications. Briefly, biopsy specimens were minced and trypsinized (0.05% trypsin/0.01% ethylenediaminetetraacetic acid) at 37°C for 3 hours. Cells were collected every 30 minutes, plated ($4 \times 10^4/cm^2$) on lethally irradiated 3T3-J2 cells ($2.4 \times 10^4/cm^2$), and cultured in 5% carbon dioxide and humidified atmosphere in keratinocyte growth medium: DMEM and Ham F12 medium (2:1 mixture) containing 10% fetal calf serum, insulin (5 µg/mL), adenine (0.18 mmol/L), hydrocortisone (0.4 µg/mL), cholera toxin (0.1 nM), triiodothyronine (2 nM), epidermal growth factor (10 ng/mL), glutamine (4 mmol/L), and penicillin-streptomycin (50 IU–50 µg/mL). One day after confluence, primary cultures were trypsinized, plated at a density of $4 \times 10^4/cm^2$ in the presence of lethally irradiated 3T3-J2 cells, and cultivated as above. Efficiency of keratinocyte colony formation was evaluated as described previously.[28]

To evaluate the melanocyte-keratinocyte ratio, dopa reaction was performed as described previously[30] on parallel primary and secondary cultures seeded in 24-well plates. In both cases, dopa reaction was performed 1 day after cells reached confluence, and the melanocyte-keratinocyte ratio was evaluated under the microscope either on dissociated cells or on epidermal sheets as described previously.[29,30]

Grafts destined for transplantation were prepared from secondary cultures 1 day after confluence, 18 to 21 days after biopsy. Briefly, cultures were washed twice in DMEM containing glutamine (4 mmol/L) and a mixture of of penicillin-streptomycin (50 IU–50 µg/mL) detached from the surface vessel with the neutral protease Dispase II (2.5 mg/mL) (Boehringer Ingelheim, Mannheim, Germany) as described previously,[21] mounted basal side up on sterile petrolatum gauze, and placed in sterile, biocompatible, and non–gas-permeable 50-cm^2 polyethylene boxes containing DMEM, glutamine (4 mmol/L), and penicillin-streptomycin (50 IU–50 µg/mL). Boxes were equilibrated at 37°C in 5% carbon dioxide humidified atmosphere for 20 minutes, thermosealed, and transferred to the hospital.

SURGICAL PROCEDURE

Achromic lesions were photographed, at times with the aid of a Wood lamp. Lesions were then outlined with a surgical marking pen, and markings were transferred to a transparent film for further semiautomatic image analysis. The recipient areas were then cleaned with povidone-iodine solution and carefully rinsed with a sterile saline solution. Local anesthesia was performed with 1% to 2% mepivacaine hydrochloride (Carbocaine). Achromic epidermis was then removed by programmed diathermosurgery (Timedsurgery), as previously described.[37,38] The Timedsurgery device (Korpo SRL, Genoa, Italy) allows precise control of all operational parameters (electric power, appropriate shape of the output waveform, emission time, dimension of the electrode). For our purpose, the apparatus was set at 1 W using the dull portion of a 0.2-mm electrode bent at an angle, and the electrode was skimmed over the skin surface (**Figure 1**C). The coagulated epidermis was then gently removed with the same electrode, but without electric power. In all vitiligo patients, achromic epidermis was removed carefully following the boundary line between the vitiligo lesion and the unaffected epidermis. When needed, the procedure was done with the aid of a magnifier.

After removal of the epidermis, the receiving bed was rinsed with sterile saline solution and covered with cultured epidermal autografts. Grafts were secured and immobilized only by dressings, consisting of one layer of Vaseline gauze (Adaptic; Johnson & Johnson Medical Inc, Arlington, Tex), followed by several layers of dry gauze and traditional bandages. After 1 week, the Adaptic gauze was replaced by Silicon N/A gauze bandages (Johnson & Johnson Medical Ltd, Ascot, England), which were worn for additional week.

Patients were then advised not to expose treated areas to detergents, creams, or perfumes for 1 month. Sun exposure was avoided for at least 3 months. Follow-up was carried out 3, 6, 9, 12, 24, and 36 months after transplantation. Repigmented areas were outlined onto transparent films 12 months after grafting and compared with the outlining done before therapy. The percentage of repigmentation was calculated using a semiautomatic image analysis system (Kontron Elektronic Imaging System KS 300; Kontron Embedded Computers AG, Eching, Germany).

cal therapy entails melanocyte transplantation and is indicated when medical therapy fails, and it could actually be considered the first therapeutic choice for the treatment of stable vitiligo. Several methods of autologous melanocyte transplantation have been developed, including suction blister grafts, thin split-thickness skin

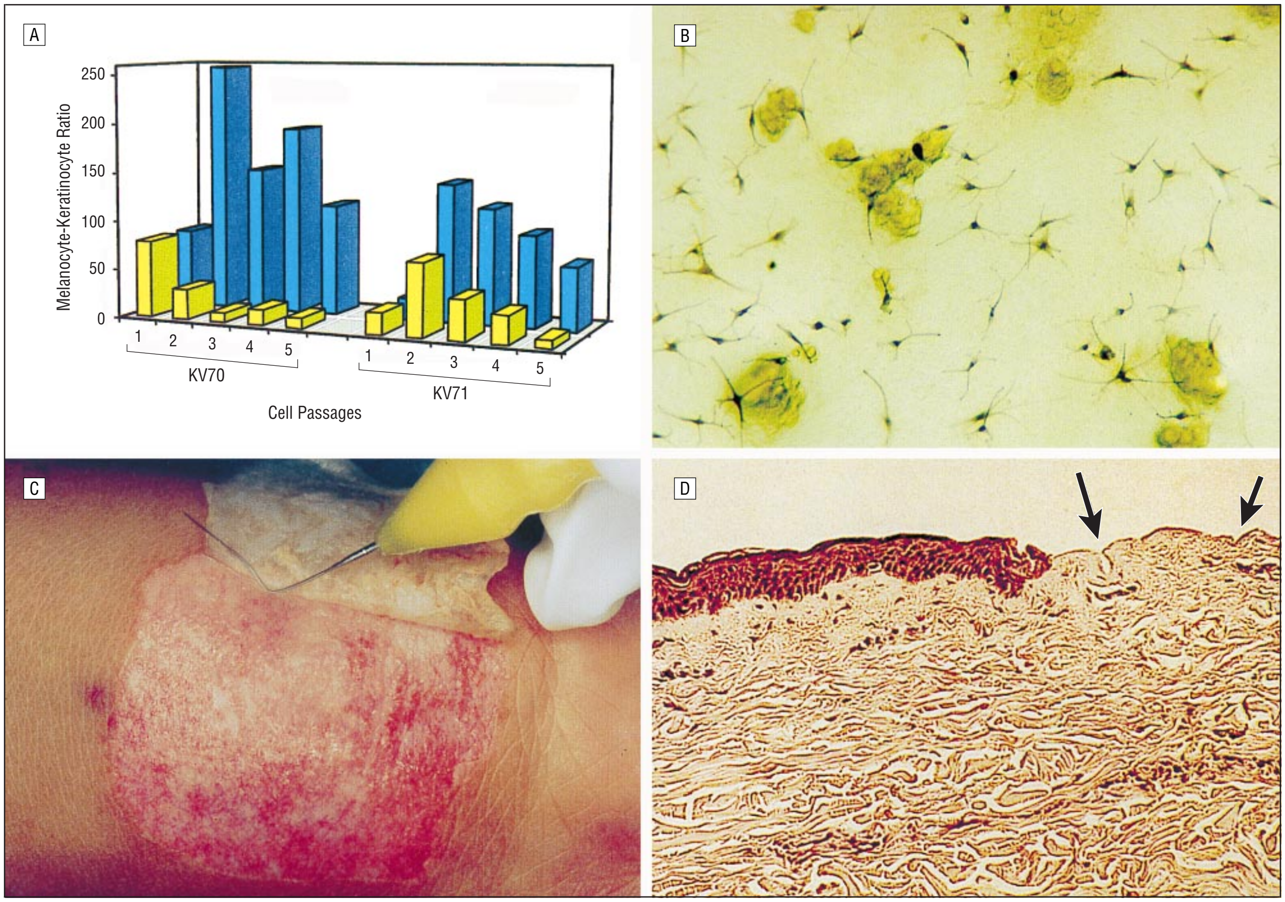

Figure 1. *A, Optimization of melanocyte-keratinocyte coculture. Epidermal cells were isolated from 2 biopsy skin specimens (strains KV70 and KV71) and cultured as described in the "Patients, Materials, and Methods" section. The melanocyte-keratinocyte ratio was evaluated by dopa reaction at each cell passage during serial cultivation. Data shown in the yellow bars are for cells seeded at a density of $7.5 \times 10^3/cm^2$ and passaged at subconfluence. Data shown in the blue bars are for cells seeded at a density of $4 \times 10^4/cm^2$ and passaged 1 day after confluence. Similar values were obtained with 2 other strains examined (not shown). B, Dopa reaction was performed on confluent epidermal sheets ready for grafting. Cells were cultivated in the same manner as for the blue bars in Figure 1A. Numerous well-differentiated melanocytes are present on the basal aspect of the epidermal sheet. C, Removal of the epidermis using the Timedsurgery device (Korpo SRL, Genoa, Italy). Figure D, Skin after the Timedsurgery procedure, showing absence of the epidermis and integrity of the dermal papillae (arrows).*

grafts, and minigrafting.[17,18] Complications of these commonly adopted surgical methods can lead to the appearance of a cobblestoned surface, spotty pigmentation, or lack of pigmentation of the treated areas, as well as to scarring of the donor sites.[17]

A potential improvement in surgical procedure could derive from cell therapy, an emerging therapeutic strategy aimed at replacing or repairing severely damaged tissues with cultured cells.[19] The main advantage of cell therapy is the possibility of producing a large amount of tissue from a small biopsy specimen. For instance, in vitro, normal human keratinocytes generate cohesive sheets of epithelium[20,21] that maintain the characteristics of the original donor site[22,23] and retain stem cells, that is, cells with extensive proliferative self-renewal capacity.[24,25] Autologous cultured epithelial sheets are routinely used for the permanent coverage of massive full-thickness burns and large mucosal defects.[26-28]

The use of autologous epidermal cultures bearing melanocytes[29,30] has also been proposed for the surgical treatment of stable vitiligo.[31-36] This technique, although promising, is still in its embryonic stage, since the number of patients treated is very limited.[18] Moreover, data concerning the amount of melanocytes transplanted, the reproducibility of the technique, and the long-term clinical results are inconclusive.[17,18] We therefore sought to evaluate the effectiveness of autologous cultured epidermal grafts bearing a controlled number of melanocytes in the surgical treatment of stable vitiligo. We report an optimized method to prepare large quantities of cultured epidermal grafts bearing a physiological number of melanocytes as well as the clinical results obtained with the application of such grafts onto 105 distinct achromic lesions (in 32 patients) prepared by means of programmed diathermosurgery (Timedsurgery).

RESULTS

OPTIMIZATION OF CULTURE CONDITIONS

Human epidermis contains approximately 4×10^6 keratinocytes per square centimeter.[39] Under trypsinization conditions allowing optimal epidermal cell yield (3×10^6 to 4×10^6 cells/cm^2), we noticed that an appropriate melanocyte-keratinocyte ratio was reproducibly maintained in culture only if the size of the skin biopsy specimen was greater than 0.25 cm^2 (data not shown). Thus, in all patients enrolled in this study, the size of the biopsy speci-

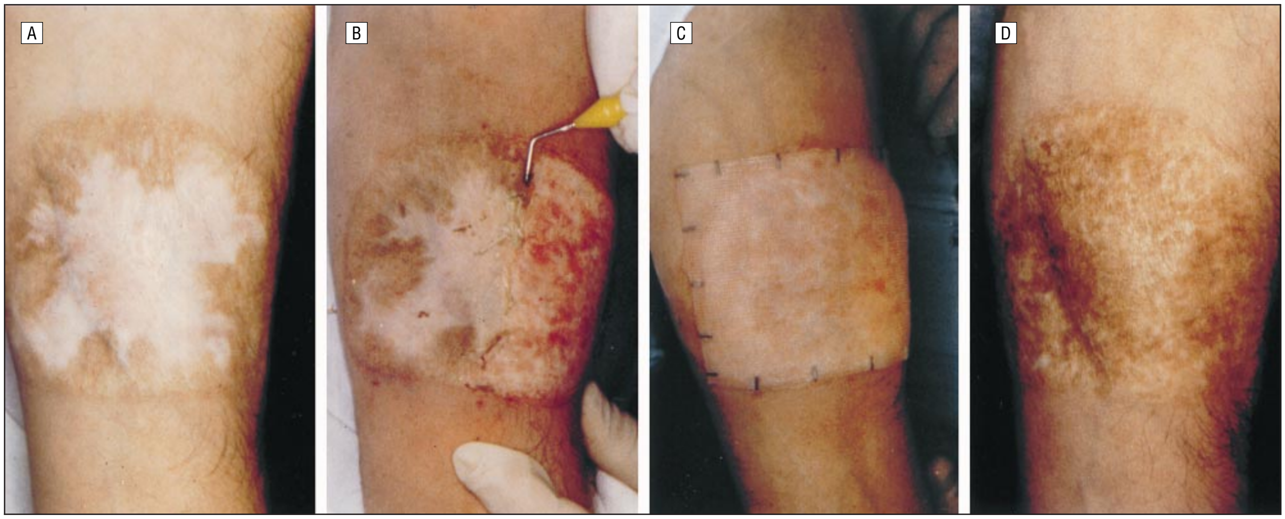

Figure 2. *The achromic epidermis of a full-thickness scar that originated from the removal of a tattoo (A) was removed using the Timedsurgery device (B) and covered with autologous epidermal cultures (C). Complete repigmentation was observed at the 6-month follow-up (D).*

men ranged from 0.5 cm^2 to 4 cm^2, according to the percentage of body surface to be treated. In preliminary experiments, we also determined that the appropriate melanocyte-keratinocyte ratio was maintained when primary keratinocytes were seeded at a density of 4×10^4 cells/cm^2. Maintenance of the appropriate melanocyte-keratinocyte ratio was independent of the body site from which the biopsy specimen was taken.

It has been reported that melanocytes in cultured epidermal grafts are depleted with serial cultivation.[40] Indeed, when primary keratinocytes were serially cultivated under conditions usually adopted for the preparation of epidermal grafts destined for full-thickness burns (ie, plated at a density of 7.5×10^3 cells/cm^2 and passaged during the exponential phase of growth),[28] a significant number of melanocytes were preserved only in primary and secondary cultures, and melanocyte concentration rapidly decreased during serial cultivation (Figure 1A, yellow bars). However, if keratinocytes were subcultivated 1 to 2 days after they reached confluence and seeded at a cell density of at least 4×10^4 cells/cm^2, the melanocyte/keratinocyte ratio was several-fold higher (Figure 1A, blue bars) and was preserved also during repeated subcultivation. In particular, the melanocyte-keratinocyte ratio observed in secondary cultures, ie, in cultures eventually destined for transplantation, was particularly favorable (Figure 1A, blue bars). Indeed, the dopa reaction clearly showed numerous melanocytes uniformly distributed in the basal layer of the cultured epidermal sheet (Figure 1B).

PREPARATION OF THE WOUND BED BY TIMEDSURGERY

The Timedsurgery device has turned the hitherto empirical electrosurgical techniques into a standardized and reproducible procedure.[37,38] By using a specific program (see the "Patients, Materials, and Methods" section), it is possible to selectively remove large areas of the epidermis from the underlying dermis using local anesthesia[37,38] and without bleeding or significant inflammation (Figure 1C). Histological examination performed after the Timedsurgery procedure shows the complete removal of the entire epidermal layer and the maintenance of the integrity of dermal papillae (Figure 1D, arrows), suggesting that removal of the epidermis by Timedsurgery allows subsequent epidermal regeneration to occur in the absence of scars. In our opinion, the absence of bleeding and inflammation and the preservation of dermal papillae represent substantial improvements over classic dermabrasion procedures.[37,38]

REPIGMENTATION OF LEUKODERMA AND STABLE VITILIGO BY MEANS OF CULTURED EPIDERMAL AUTOGRAFTS

In preliminary experiments, we sought to investigate whether cultured epidermal autografts bearing a physiological number of melanocytes were able to induce repigmentation in leukodermal lesions unequivocally devoid of melanocytes and hair follicles. A 50-cm^2 full-thickness achromic scar originating from the removal of a tattoo (**Figure 2**A) was prepared by Timedsurgery (Figure 2B) and covered with autologous epidermal cultures (Figure 2C). Complete repigmentation was observed (Figure 2D), strongly suggesting a "take" of the melanocytes present in the grafts. This prompted us to investigate the use of autologous epidermal cultures to treat stable vitiligo.

Figure 3 shows 2 patients suffering from generalized (Figure 3A-3C) and focal (Figure 3D-3F) vitiligo before transplantation (Figure 3A and Figure 3D, respectively) and 18 and 12 months after transplantation (Figure 3B-3C and Figure 3E-3F, respectively) of cultured epidermal autografts. As shown in Figure 3B and Figure 3E, complete repigmentation was obtained in the absence of scars. With the use of the Wood lamp (Figure 3C and Figure 3F), it was possible to identify in both patients the margin of the transplanted areas, which was still depleted of melanocytes (Figure 3C and Figure 3F, at arrows). This strongly suggests that repigmentation was attained through the engraftment of me-

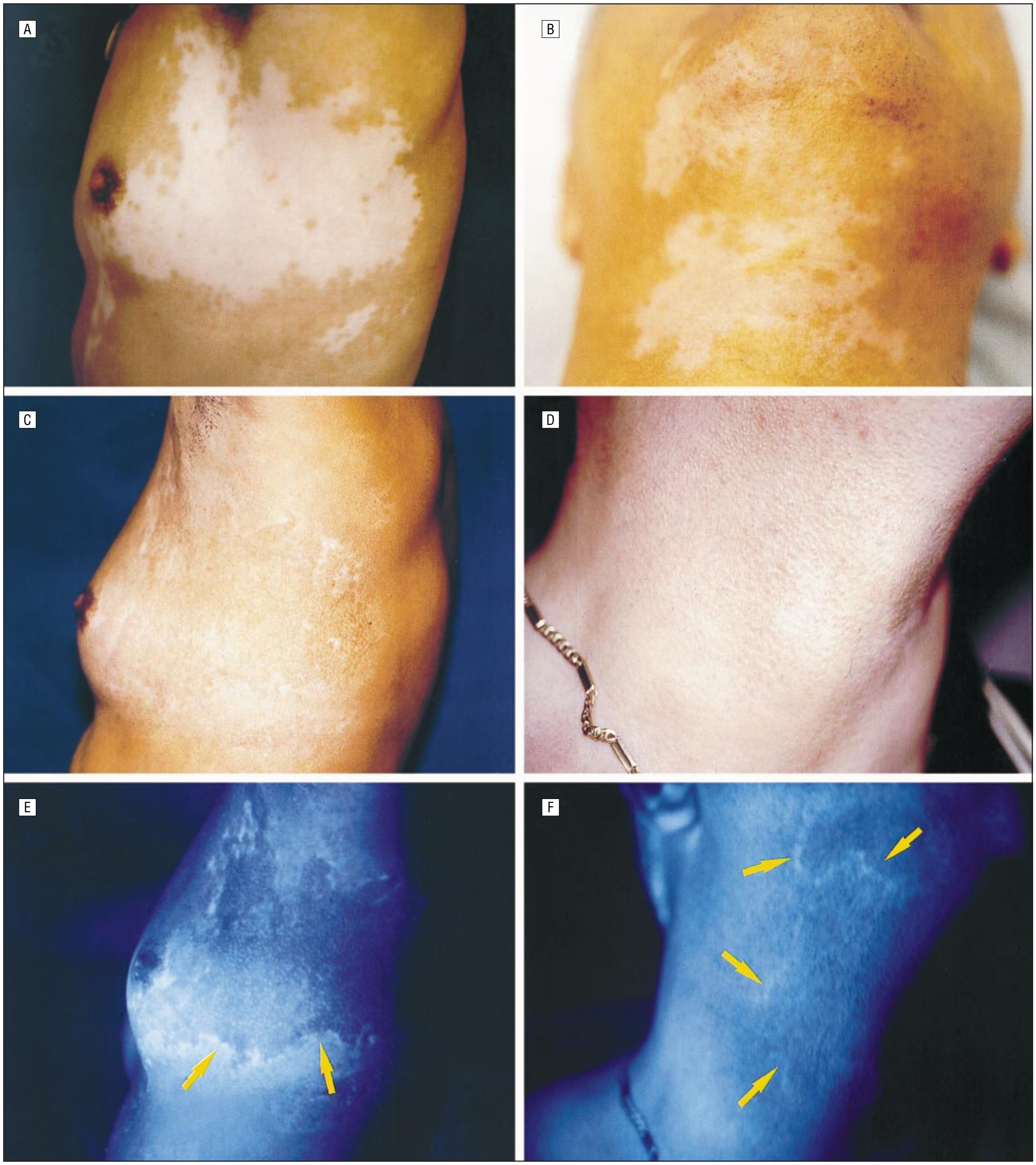

Figure 3. *Achromic lesions of 2 patients suffering from generalized (A) and focal (B) vitiligo were treated using the Timedsurgery device followed by the application of cultured epidermal grafts. Complete repigmentation was observed after 18 (C) and 12 (D) months of follow-up, respectively, with no scars. Examination under a Wood lamp (E and F, respectively) confirmed complete repigmentation. Note, however, that the edges of both lesions were still depleted of melanocytes (E and F, arrows).*

lanocytes present in the cultured epidermal sheets and not through migration of melanocytes surrounding the achromic lesions.

Figure 4A and 4B show a patient with segmental vitiligo affecting the chin and the neck before and after epidermal repigmentation obtained by the application of epidermal cultures. In this patient, achromic hairs (Figure 4A, arrow) remained achromic after grafting (Figure 4B, arrows), which suggests that, at least in this patient, epidermal repigmentation was obtained through engraftment of cultured melanocytes and not through the migration of melanocytes from hair follicles, and that the engraftment of epidermal melanocytes was not sufficient to restore pigmentation of achromic hairs. Notably, it has been reported that repigmentation of achromic vellus hairs within the vitiliginous skin can indeed

Figure 4. *A and B, The chin and the neck of a patient presenting with segmental vitiligo (A) were treated with epidermal cultures. Complete repigmentation was obtained in the absence of scars (B). Note that achromic hairs (A, arrow) remained depigmented after grafting (B, arrows), suggesting that engrafted melanocytes were not able to repopulate hair follicles. C and D, Both legs of a patient presenting with "stable" generalized vitiligo (C) were treated with cultured epidermal sheets applied with a single operation. Complete and stable repigmentation was obtained with no scars (D). E and F, Nipples and areolae of a patient with generalized vitiligo (E) were treated with epidermal cutures. Melanocyte engraftment yielded a skin color (F) indistinguishable from that of a normal healthy control.*

Table 1. Treatment of Vitiligo by Timedsurgery and Transplantation of Cultured Epidermal Autografts

Patient No./Phototype/ Age, y/Sex	Clinical Type of Vitiligo	Melanocyte-Keratinocyte Ratio on Cultured Autografts	Treated Area, cm^2	Repigmentation, %	Follow-up, mo
1/III/50/F	Acrofacial	1:405	60	98.3	36
2/IV/43/M	Acrofacial	1:412	180	68.9	33
3/IV/33/M	Acrofacial*	1:135	240	0	29
4/IV/22/M	Generalized	1:131	194	100	29
5/III/31/M	Generalized	1:318	1566	99.9	29
6/III/50/M	Focal	1:180	115	100	25
7/II/11/M	Segmental	1:178	280	98	24
8/III/21/F	Acrofacial*	1:150	54	22.6	24
9/IV/29/M	Focal	1:140	75	98.4	24
10/III/19/M	Focal	1:217	75	95	24
11/IV/22/M	Generalized	1:159	53	0	24
12/IV/45/F	Acrofacial	1:31	100	0	23
13/IV/30/F	Generalized	1:58	700	98	23
14/III/28/F	Segmental	1:39	400	80	22
15/III/36/F	Acrofacial	1:81	90	7	21
16/II/63/M	Generalized	1:135	176	0	21
17/IV/21/F	Generalized	1:123	375.2	100	21
18/V/29/F	Acrofacial*	1:156	71	50	20
19/III/38/F	Generalized	1:169	65	73.8	20
20/IV/14/F	Generalized	1:100	172	43.5	20
21/III/22/F	Focal	1:130	14	100	19
22/III/38/F	Focal	1:370	59	76.3	18
23/V/37/M	Focal	1:327	16	95	18
24/III/40/F	Generalized*	1:99	120	40	18
25/III/71/M	Segmental	1:114	60	98	17
26/III/20/F	Focal*	1:124	32	36.2	17
27/II/22/M	Acrofacial	1:85	61.5	23.1	13
28/V/22/M	Generalized	1:143	79	89.0	13
29/III/28/F	Generalized*	1:225	320	40	12
30/III/32/M	Focal	1:131	90	95	12
31/IV/40/M	Segmental	1:80	90	100	12
32/III/34/F	Generalized*	1:155	95.5	56.0	12

**New lesions appeared during follow-up.*

occur through recolonization of the hair bulb by melanocytes present in cultured epidermal sheets.[34,36] Our data suggest, however, that this is not a general rule.

Figure 4C through Figure 4F also show 2 patients suffering from generalized vitiligo. Both legs of the first patient (Figure 4C) were treated with cultured epidermal autografts applied with a single operation. Complete and stable repigmentation was obtained in the absence of scars (Figure 4D). The second patient had vitiligo on the chest, including the nipples and areolae (Figure 4E), and was severly distressed by this. After grafting of epidermal cultures, the chest (not shown), nipples, and areolae (Figure 4F) were stably repigmented.

Interestingly, even if epidermal cells were cultivated from a biopsy specimen taken from the pubis, pigmentation of the nipples and areolae was indistinguishable from that of a normal healthy control, suggesting that underlying dermis can play an important role in modulating melanocyte activity in vivo.

EVALUATION OF THE CLINICAL PERFORMANCE OF CULTURED EPIDERMAL GRAFTS

Thirty-two patients suffering from different types of vitiligo were treated with autologous epidermal cultures (**Table 1**). At admission, all patients were judged to have stable vitiligo. All patients did not respond to several therapeutic attempts made with conventional medical methods and did not receive any therapy for at least 12 months. All patients were treated exclusively with cultured epidermal grafts; that is, they had not received any additional medical (including UV radiation) or surgical therapy after transplantation or during the entire follow-up. To ensure that cultured epidermal grafts contained the appropriate number of melanocytes, the melanocyte-keratinocyte ratio was monitored for each patient on a randomly chosen graft the day before surgery. As shown in Table 1, the melanocyte-keratinocyte ratio ranged between 1:31 and 1:412.

A total of 6078.2 cm^2 of body surface was grafted (Table 1). Final evaluation of the percentage of repigmentation was performed after at least 12 months, and stability of repigmentation was evaluated during 12 to 36 months of follow-up. A total of 4667.9 cm^2 of body surface, corresponding to 77% of the treated areas, was fully and stably repigmented (Table 1). Variation of the melanocyte concentration in cultured grafts within the above range (1:31 to 1:412) was not correlated with the percentage of final repigmentation or with the intensity of repigmentation. Indeed, in all patients who had achieved repigmentation, the color of the treated areas was similar to that of the uninvolved surrounding skin,

Table 2. Treatment of "Stable" Vitiligo by Timedsurgery and Transplantation of Cultured Epidermal Autografts (Followup of 12-36 mo)

	All Patients	Patients With Positive Results (71%-100% Repigmentation)	Patients With Negative Results (0%-70% Repigmentation)
No. of treated patients	**25**	18	7
Female/male, No.	**10/15**	7/11	3/4
Mean age, y	33.4	32.8	35.0
Clinical type of vitiligo			
Localized focal	**7**	7	0
Localized segmental	**4**	4	0
Generalized vulgaris	**9**	6	3
Generalized acrofacial	**5**	1	4
No. of treated lesions	**75**	44	31
Area of transplanted autografts, cm^2	**5146**	4313	833
Repigmentation, % (repigmented/transplanted, cm^2)	85.1 (4379.15/5145.7)	96.4 (4159.85/4313.2)	26.3 (219.3/832.5)

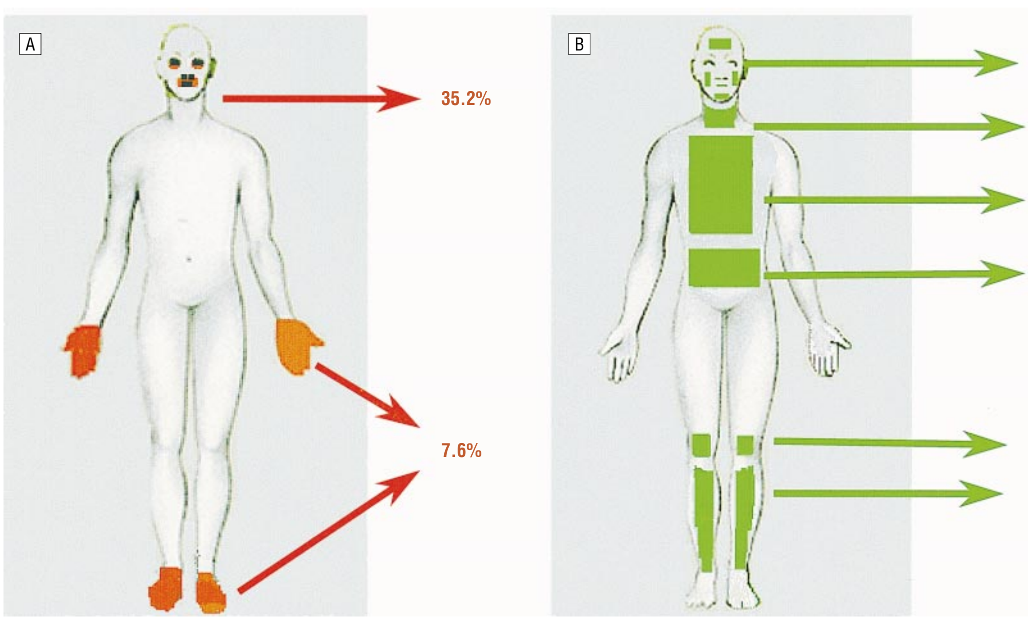

Figure 5. *Average percentage of repigmentation observed in different body sites of patients suffering from "stable" vitiligo and treated with cultured epidermal sheets.*

even after sun exposure. In all patients, scar formation was never observed.

It is worth noting that in a few patients (Table 1), new achromic lesions appeared after 6 to 9 months of follow-up, suggesting either reactivation of the disease or an erroneous first diagnosis of stable vitiligo. As expected, the average percentage of repigmentation in these patients was only 31%.

In contrast, as shown in **Table 2**, the average percentage of repigmentation in the remaining 25 patients was 85% (4379.1 cm^2 repigmented/5145.7 cm^2 transplanted). In these patients, repigmentation obtained after a single operation was either very satisfactory (70% to 100% of the treated areas) or very disappointing (0% to 30% of the treated areas), without intermediate clinical outcomes (Table 1). We therefore arbitrarily expressed data simply as positive (71% to 100% repigmentation of the treated areas) or negative (0% to 70% repigmentation of the treated areas) clinical results. As shown in Table 2, the percentage of repigmentation was 96% of the treated areas (4159.8 cm^2 repigmented/4313.2 cm^2 transplanted) in 18 patients showing positive results and of only 26% of the treated areas (219.3 cm^2 repigmented/832.5 cm^2 transplanted) in 7 patients showing negative results. All patients suffering from focal and segmental vitiligo showed complete and stable repigmentation, while most of the patients with acrofacial vitiligo had very poor clinical results. An intermediate situation was observed with generalized vitiligo vulgaris.

Since this variability could be only partially explained by the "activity" of vitiligo and was only partially related to its clinical type, we analyzed the data as a percentage of repigmentation obtained in specific body sites independent of the clinical type of the disease. As shown in **Figure 5**, poor clinical results were obtained in the upper and lower extremities, as well as in periorificial areas (Figure 5A). In contrast, excellent results were obtained in all the other body sites treated, including the face (Figure 5B). Indeed, stable repigmentation ranged from 88% to 96% of the treated areas.

In summary, our data show that autologous cultured epidermis can induce steady and complete (over 90%) repigmentation of stable vitiligo (4329.7 repigmented cm^2/4675.2 transplanted cm^2), although this technology does not appear to be useful in the treatment of achromic lesions of hands, feet, and periorificial body sites or active vitiligo.

COMMENT

Transplantation of cultured melanocytes has been proposed as a possible, although still experimental, alternative to conventional surgical methods for the treatment of stable vitiligo.[17,18] Cultured melanocytes can be inoculated as a pure cell suspension[41-46] or in co-culture with keratinocytes, as with cultured epidermal sheets.[29-36] Several considerations convinced us that the use of cultured epidermal sheets is more appropriate than pure melanocyte cultures: (1) Keratinocytes regulate melanocyte growth and differentiation, as well as the proper melanocyte-keratinocyte ratio.[29,30] (2) Melanocytes organize themselves into the basal layer of the cultured epidermis, develop dendritic arborization with melanosome-containing processes, and transfer melanosomes into basal keratinocytes,[29,30,47] and hence maintain their physiological characteristics when co-cultured with keratinocytes. (3) Keratinocyte cultivation allows the easy production of large quantities of cultured autografts (up to 2 m^2)[21,28] in a shorter time than that required for pure melanocyte cultivation. (4) Most importantly, cultured epidermal grafts have been widely used worldwide for 20 years for the treatment of thousands of patients suffering from large skin and mucosal defects,[26-28,48,49] and there has never been an increased risk of either carcinoma or melanoma reported. This last consideration is of particular relevance, since the remote possibility of undesired tumorigenic risks due to cultivation has so far limited the use of pure melanocyte cultures.[17,18]

This said, the data available on the use of cultured epidermal grafts are still very limited,[18] banishing this technology at a developmental stage quite far from routine daily practice.

In this article, we show that cultured epidermal grafts can indeed be considered as a therapeutic alternative to other proposed surgical techniques. Our data also suggest that repigmentation can be obtained by means of true "take" of cultured melanocytes, as opposed to migration (potentially induced by surgical maneuvers) of resident melanocytes from surrounding skin or from hair follicles. Obvious advantages of epidermal cultures are the possibility of transplanting a large body surface using local anesthesia and a single operation, as well as the complete absence of scar formation, which can also be explained by the reproducible accuracy of the noninvasive disepithelialization attained by means of the Timedsurgery procedure.

A key issue for the successful clinical outcome of cell therapy deals with the quality control of the culture system. For instance, unsatisfactory epidermal regeneration, which has been reported with the use of cultured epidermal autografts in full-thickness burns,[28,49] might arise from the depletion of epidermal stem cells, which can occur because of incorrect culture conditions or inappropriate cell substrates.[28] Similarly, culture conditions should be optimized for the application of cultured grafts to be used in stable vitiligo. In this case, the maintenance of the proper melanocyte concentration within the epidermal grafts is the most important quality control. Therefore, in our opinion, the melanocyte-keratinocyte ratio should be routinely evaluated in cultures before grafting.

Our data show that the success rate of cultured epidermal autografts can be comparable to the highest success rate usually achieved by split-thickness skin grafts or epidermal blister grafts.[18] However, while the success rate was high in most of the body sites (Figure 5B), we were unable to obtain substantial improvement of achromic lesions of the upper and lower extremities or of periorificial areas of the face. This is disappointing, since patients affected by vitiligo usually complain about leukoderma in these exposed body sites. We are currently investigating whether these poor clinical results stem from an intrinsic refractoriness of these body sites to surgical therapy, or whether poor results could be explained by the difficulty in immobilizing hands, feet, and periorificial areas, hence hampering the "take" of melanocytes to the skin graft.

We also noticed the appearance of new lesions in some of our patients, suggesting a reactivation of the disease (or an erroneous first diagnosis of stable vitiligo). Obviously, in these patients, clinical results were not satisfactory. These problems could be at least partially circumvented by the minigraft test, as suggested by Falabella and colleagues.[50] In our experience, however, very few patients were favorably disposed toward the minigraft test; hence, we were unable to perform this test regularly. An alternative comes from data published by Njoo et al,[14] showing the association of the Koebner phenomenon with the activity of the disease. We are currently developing cryopreservation procedures allowing maintenance of melanocyte viability. This should give the possibility of planning cell grafting 6 months after biopsy in order to evaluate the appearance of the Koebner phenomenon at the site from which the biopsy specimen was taken.

Finally, disadvantages of the technology described in this article relate to the complexity of the culture system, to the high level of expertise required for the maintenance of the proper quality of the grafts, and to the high cost of the cultures. However, the good clinical results that can be obtained by cultured epidermal grafts should prompt investigation aimed at finding solutions to these problems and improving disepithelialization techniques in order to allow the coverage of larger body surface areas in a shorter time and at a reasonable cost.

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