Erbium:YAG Laser and Cultured Epidermis in the Surgical Therapy of Stable Vitiligo

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Objective: To induce complete and reproducible repigmentation of large "stable" vitiligo lesions by means of autologous cultured epidermal grafts using a rapid, simple, and minimally invasive surgical procedure.

Design: Achromic epidermis was removed by means of appropriately settled erbium:YAG laser, and autologous epidermal grafts were applied onto the recipient bed. Melanocyte content was evaluated by dopa reaction. The percentage of repigmentation was calculated using a semiautomatic image analysis system.

Setting: A biosafety level 3–type cell culture facility, a surgical ambulatory department, and a dermatological department in a hospital.

Patients: Twenty-one patients with different types of vitiligo were admitted to the study and treated with autologous cultured epidermal grafts. Inclusion criteria were failure of at least 2 standard medical approaches; no therapy for at least 12 months; no progression of old lesions or appearance of new lesions; no Koebner phenomenon within the past 18 months; and no autoimmune disorders.

Results: The average percentage of repigmentation in 21 patients was 75.9% (1759.7 cm^2 repigmented/2315.8 cm^2 transplanted). Three patients showed a reactivation of their vitiligo and did not show repigmentation. The remaining 18 patients, with 43 distinct lesions, showed an average percentage of repigmentation of 90% (1759.7 cm^2 repigmented/1953.4 cm^2 transplanted).

Conclusions: Under appropriate conditions, cultured epidermal grafts induce complete repigmentation of stable vitiligo lesions. Erbium:YAG laser surgery can supply a fast and precise tool for disepithelialization, hence allowing treatment of large vitiligo lesions during a single surgical operation.

Arch Dermatol. 2003;139:1303-1310

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OUR GROUP has shown that autologous cultured epidermis can induce a steady and complete repigmentation of "stable" vitiligo.[1] The success rate of cultured epidermal autografts[1] is comparable to the highest success rate achieved by split-thickness skin grafts or epidermal blister grafts,[2-4] provided that appropriate culture conditions are adopted.[1] However, epidermal cultures perform poorly on achromic lesions of fingers and periorificial (perioral and periocular) areas.[1] Advantages of epidermal culture transplantation are the ability to treat a large body surface area using a small skin biopsy specimen and the absence of scar formation, which can also be explained by the less invasive disepithelialization procedure achieved by means of programmed diathermosurgery (timedsurgery).[1] Indeed, timedsurgery allows precise removal of the epidermis and preservation of the papillary dermis[1,5] without bleeding and inflammation.

However, timedsurgery is a time-consuming procedure, and so the width of the area that can be treated during a single surgical operation is limited. This prompted us to improve the disepithelialization technique to graft larger body surface areas in a shorter time while maintaining the accuracy of epidermal removal without bleeding and inflammation. Among the different known disepithelialization procedures,[4,6-19] we chose laser technology. Among the multiple laser-based techniques available, erbium:YAG (Er:YAG) lasers[12-15] and carbon dioxide lasers[16-19] have been used to remove the epidermis in the surgical treatment of vitiligo with either conventional procedures[12-17] or with pure melanocyte cultures.[18,19]

The Er:YAG laser was introduced into laser resurfacing procedures as an alternative to carbon dioxide laser surgery in the hope of maintaining clinical benefits and decreasing adverse effects.[20] Indeed, Er:YAG allows precise tissue ablation with minimal coagulative damage.[20] Herein we evaluate the effectiveness of transplanta-
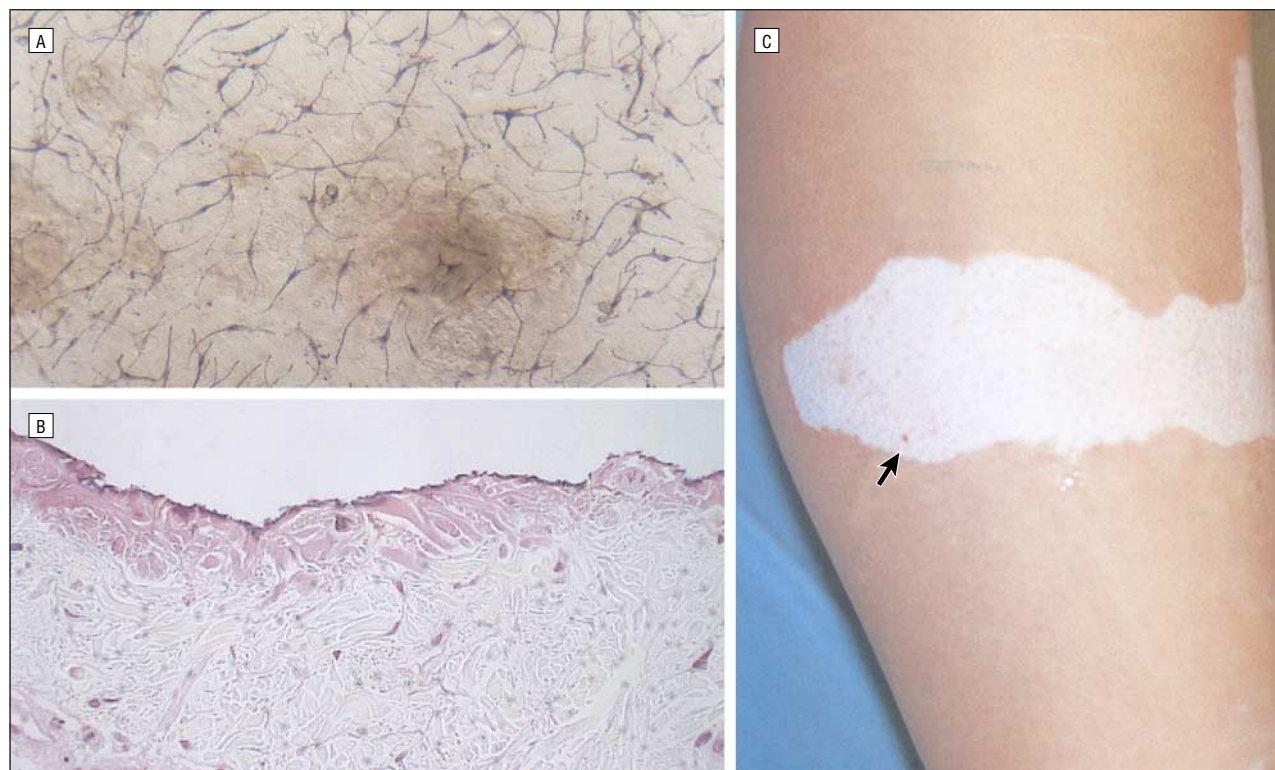
Figure 1. A, Results of a dopa reaction performed on a parallel confluent epidermal sheet (original magnification ×10). B, Histologic specimen taken from leg skin after erbium:YAG laser surgery at a fluence of 15.9 J/cm². Note that the epidermis has been completely removed, and the dermal papillae are well preserved. The limited thermal damage on the surface of the papillary dermis did not interfere with a satisfactory graft (hematoxylin-eosin, original magnification ×40). C, Patient's leg after removal of the epidermis by means of erbium:YAG laser surgery. Disepithelialization was performed by ablating slightly less than what was necessary to achieve pinpoint bleeding (arrow).

tion of autologous cultured epidermal grafts bearing a controlled number of melanocytes on Er:YAG laser–prepared achromic lesions in 21 patients with different forms of vitiligo.

METHODS

INCLUSION CRITERIA

Patients were evaluated clinically, by a questionnaire, and by serologic tests; patients presenting with autoimmune disorders (thyroid disease, diabetes mellitus, alopecia areata, pernicious anemia, or 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) no progression of old lesions within the past 18 months; (4) no new lesions developing within the past 18 months; and (5) no Koebner phenomenon within the past 18 months.

Twenty-one patients (with 49 distinct achromic lesions) with different types of vitiligo were enrolled in the study. The duration of clinical stability ranged from 18 months to 12 years. Informed consent was obtained from all patients. Procedures were in accordance with the ethical standards of the committees on human experimentation of our institutions.

CELL CULTURE

3T3-J2 cells were cultured in Dulbecco-Vogt Eagle medium containing 10% calf serum, 4mM glutamine, and combination of 50 IU of penicillin and 50 µg/mL of streptomycin. Full-thickness (0.5-3.3 cm²) skin biopsy specimens were taken from unaffected and, when possible, hairy body areas. Keratinocytes and melanocytes were cultured as previously described.[1,21] Briefly, biopsy specimens were minced and trypsinized (0.05% trypsin/0.01% edetic acid) at 37°C for 3 hours. Cells were collected every 30 minutes, plated (4×10^4/cm²) on lethally irradiated 3T3-J2 cells (2.4×10^4/cm²), and cultured in 5% carbon dioxide and humidified atmosphere in keratinocyte growth medium: Dulbecco-Vogt Eagle medium and Ham F12 media (2:1 mixture) containing 10% fetal calf serum, 5 µg/mL of insulin, 0.18mM adenine, 0.4 µg/mL of hydrocortisone, 0.1nM cholera toxin, 2nM triiodothyronine, 10 ng/mL of epidermal growth factor, 4mM glutamine, and combination of 50 IU of penicillin and 50 µg/mL of streptomycin.

One day after confluence, primary cultures were trypsinized, plated at a density of 4×10^4/cm² in the presence of lethally irradiated 3T3-J2 cells, and cultivated as above.

The keratinocyte colony-formation efficiency (CFE) was determined by plating 1000 cells, fixing colonies with 3.7% formaldehyde 12 to 14 days later, and staining them with 1% rhodamine B. Total and aborted colonies were evaluated with the aid of a phase-contrast microscope. Total colonies were calculated as a percentage of total plated cells; aborted colonies (small colonies with irregular shape and terminally differentiated squamelike keratinocytes)[22] were calculated as a percentage of total colonies.

Dopa staining was performed on parallel primary and secondary cultures seeded in 24-multiwell plates.[23,24] Briefly, cultures were fixed in buffered formalin; the plasma membrane was permeabilized for 5 minutes in 0.5% Triton X-100 (20mM HEPES [pH 7.4], 300mM sucrose, 50mM sodium chloride, and 3mM magnesium chloride), and dopa reaction was carried out for

Table 1. Treatment of Vitiligo by Erbium:YAG Laser Surgery and Transplantation of Cultured Epidermal Autografts: Clinical Data of 21 Patients

Patient No.	Skin Phototype/Age, y/Sex	Clinical Type of Vitiligo	Onset/Stability of Vitiligo, mo	Treated Body Areas	W-mJ-Hz (No. of Laser Passes)	Fluence, J/cm²	Treated Areas, cm²	Repigmented Areas, cm²	Repigmentation, %	Last Follow-up, mo
1	IV/32/F	Generalized	300/144	Arm (internal area)	1-200-5 (1)	6.3	28	28	100	42
2	III/34/M	Generalized	348/72	Legs	4-400-10 (1)	12.7	105	105	100	32
3	IV/16/F	Generalized	120/18	Knees-legs	5-500-10 (1)	15.9	130.9	130.9	100	31
				Ankles-feet	5-500-10 (1)	15.9	195.8	195.8	100	16
4	III/16/M	Focal	96/96	Abdomen	2-200-10 (1)	6.3	40	40	100	20
5	II/21/M	Generalized	132/24	Feet	5-500-10 (1)	15.9	106.3	106.3	100	15
6	II/15/F	Focal	60/48	Forehead-cheek	1-200-5 (1)	6.3	11.6	11.6	100	12
7	III/26/M	Focal	156/120	Forehead-cheek-neck	2-200-10 (1)	6.3	35	34.6	98.8	12
8	V/48/F	Generalized	120/24	Chin-neck	2-200-10 (1)	6.3	39.7	39	98.2	12
9	III/26/F	Segmental	204/24	Abdomen	1-200-5 (1)	6.3	156.6	151.9	97	12
10	III/21/F	Generalized	108/18	Left pubis-left inguinal fold	1-200-5 (1)	6.3	178.2	171.1	96	12
11	III/22/M	Segmental	228/228	Chin-neck	1-200-5 (1)	6.3	19.9	18.9	94.9	9
12	III/34/F	Segmental	108/60	Chest	1-200-5 (1)	6.3	317.5	301.6	94.9	45
13	III/22/M	Segmental	228/228	Left forehead	1-200-5 (1)	6.3	216.9	196.9	90.7	9
				Left leg	2-400-5 (1)	12.7				
14	IV/24/F	Focal	120/60	Arm (external area)	5-500-10 (1)	15.9	53.1	47.7	89.8	31
15	III/22/F	Generalized	144/24	Feet	4-400-10 (1)	12.7	96	79.9	83.2	32
16	III/15/F	Focal		Back-shoulder	1-200-5 (1)	6.3	37	27.1	73.2	9
17	V/59/M	Generalized	288/60	Hands	3-300-10 (2)	19.1	137	73.4	53.5	29
18	III/33/F	Acrofacial	276/24	Left hand	3-300-10 (2)	19.1	48.9	0	0	17
19	III/61/F	Focal	24/18	Cheeks-neck	1-200-5 (1)	6.3	46.5	0	0	9
20	III/34/F	Generalized	168/18	Right hand	3-300-10 (2)	19.1	110.5	0	0	12
				Neck	1-200-5 (1)	6.3				
21	II/22/F	Generalized	108/18	Right arm (external area)	5-500-10 (1)	15.9	205.4	0	0	9
Total	. . .	. . .	. . .	. . .	. . .	. . .	**2315.8**	**1759.7**	**75.9**	. . .

3 hours. Dopa reaction on epidermal sheets (**Figure 1**A) was performed 1 day after cells reached confluence, as previously described.[23,24] The melanocyte-keratinocyte ratio (M/K) was evaluated under the phase-contrast microscope as follows: Under the same culture conditions indicated above, we verified the findings of Barrandon and Green[22] that cell density is approximately 2000 keratinocytes/mm² (unpublished data, 1996). Thus, to evaluate the M/K, we divided the number of keratinocytes by the average number of melanocytes calculated in 10 randomly chosen 2.4 mm² areas (approximately the area seen with a ×10 magnification) of confluent cultured epidermis (a_m:a_k = 1_m:x_k, where a_m is the calculated average number of total melanocytes in 2.4 mm²; a_k is the theoretical number of total keratinocytes in 2.4 mm²; m = melanocyte; and k = keratinocyte).

Grafts destined for transplantation were prepared from secondary cultures 1 day after confluence, as previously described.[1]

Er:YAG LASER SURGERY AND APPLICATION OF CULTURED AUTOGRAFTS

Achromic lesions were photographed and outlined with a surgical marking pen. Markings were transferred to a transparent film for further semiautomatic image analysis (Kontron Elektronic Imaging System KS 300; Kontron Embedded Computers AG, Eching, Germany).

The recipient areas were cleaned with povidone-iodine and rinsed with a sterile isotonic sodium chloride (saline) solution, and the operative field was dried with cotton gauzes. Achromic epidermis was removed by using the pulsed Er:YAG laser (Laser Smart 2940; DEKA Medical Electronics Laser Associated srl, Calenzano, Florence, Italy) with a 2-mm spot–sized handpiece at a setting of 200 to 500 mJ. The fluence (the amount of energy per skin surface, expressed in joules per square centimeter)[25] ranged from 6.3 to 19.1 J/cm² (**Table 1**).

The denatured epidermal residues were removed with saline-moistened gauze. Usually, disepithelialization was performed without anesthesia.[26,27] However, with fluences greater than 10 J/cm² and in larger areas, a pretreatment with 2.5% lidocaine and 2.5% prilocaine combination (EMLA cream; AstraZeneca SpA, Basiglio, Milan, Italy) under 2-hour occlusion was performed.[26] As confirmed by histologic data (Figure 1B), complete disepithelialization was obtained by ablating slightly less than what was necessary to achieve pinpoint bleeding (Figure 1C).

One laser pass was sufficient for almost all patients (Table 1). Two laser passes were used only on hand lesions.

After disepithelialization, the receiving bed was rinsed again with sterile saline solution and covered with autologous cultured epidermal sheets. Grafts were secured and immobilized only by a dressing, as previously described.[1] Patients received a 1-week regimen of antibiotic and anti-inflammatory therapy. After 7 days, bandages and grafts were removed, and the "take" was evaluated. Patients were then advised not to expose treated areas to detergents, creams, or perfumes for 1 month. Sun avoidance was required for at least 2 to 3 months. After 2 to 3 months, only mild sun or UV-A exposure was recommended. Follow-up visits were carried out 3, 6, 9, 12, and 18 months after transplantation, and when possible 24 months, 36 months, and longer after transplantation. Definitive repigmentation was seen at the 6-month follow-up visit. Further controls were carried out only to confirm the maintenance of the achieved repigmentation. Repigmented areas were outlined onto transparent films 6 months after grafting and compared with the outlining done before therapy.

Table 2. Treatment of Vitiligo by Erbium:YAG Laser Surgery and Transplantation of Cultured Epidermal Autografts: Biological Data of Cell Cultures in 21 Patients

Patient No.	Biopsy Specimen Size, cm²/ Body Area	Cell Yield/ cm²	CFE of the Biopsy Specimen*	CFE of Confluent Primary Cultures*	CFE of Confluent Secondary Cultures*	M/K Primary Cultures	M/K in Secondary Cultures
1	1.8/Buttock	4.5×10^6	5.5/0	72.5/2.1	7.1/26.6	1:62	1:65.7
2	1/Pubis	4×10^6	2.4/0	9.6/7.6	19.4/8.2	1:101.6	1:141.4
3	3/Buttock	2.8×10^6	5.3/0	12.4/1.6	35.2/0.3	1:90.3	1:103
	2.6/Buttock	3.8×10^6	2.6/0	14.7/1	10.5/27.1	1:39.2	1:52.9
4	1.5/Pubis	3.6×10^6	5.6/0	10.5/4.3	26.6/9.1	1:94.2	1:89.6
5	1.7/Pubis	3.8×10^6	3/0	29.6/3.2	10.7/36.2	1:95.8	1:48.6
6	0.5/Pubis	5.2×10^6	5.4/0	17.5/1.1	ND	1:113.8	1:109.8
7	0.5/Pubis	2.4×10^6	7.1/0	16.7/0.2	16.2/9.5	1:41.9	1:42.1
8	ND/Pubis	ND	2.8/0	19.9/4	21/20.5	1:116.1	1:122.2
9	1.5/Pubis	4.3×10^6	8.8/0	48.2/0	ND	1:65.8	1:51.4
10	0.9/Pubis	3.6×10^6	7.4/0	21.8/0.2	15.2/23.4	1:88.3	1:80.9
11	1/Pubis	4.8×10^6	9.2/0	37.7/0.2	27.4/3.4	1:50.1	1:30.1
12	1.2/Pubis	3.6×10^6	7.5/0	26.5/0.5	10.5/26.5	1:137.9	1:102
13	2/Pubis	4.2×10^6	8.5/0	23.3/0.6	13.8/35.5	1:53.2	1:36.4
14	1.5/Pubis	3.6×10^6	7.6/0	13.7/0.3	17.2/7.7	1:64.5	1:70.1
15	1.5/Buttock	3.6×10^6	2.6/0	10.7/1.3	25.8/17.5	1:224	1:176.6
16	1/Pubis	2.7×10^6	5.5/0	ND	28.3/14.1	ND	1:87.2
17	3.3/Thigh	1.7×10^6	6.1/0	18.8/2.3	13.6/26.5	1:110.4	1:108.7
18	1.3/Pubis	3.6×10^6	3.6/0	21.8/1.1	ND	1:301	1:127.2
19	1/Pubis	2.3×10^6	2/0	6/2.4	15.2/33.6	1:94.5	1:169.2
20	1.7/Pubis	3.4×10^6	9.6/0	5.9/0.8	ND	1:294.7	1:200
21	2/Thigh	4×10^6	4/0	8.8/2.3	28.5/5.4	1:75.5	1:91.9

Abbreviations: CFE, colony-forming efficiency; M/K, melanocyte-keratinocyte ratio; ND, not determined.
*Data are total colonies/aborted colonies.

RESULTS

QUALITY CONTROLS OF CELL CULTURES

The average CFE percentages of keratinocytes isolated from skin biopsy specimens, primary cultures (1 day after confluence), and cultured grafts were 5.5% (no aborted colonies), 21.2% (1.7% aborted), and 19% (18.3% aborted), respectively (**Table 2**). It is not surprising that keratinocytes cultivated under conditions that privilege melanocyte growth (keratinocytes subcultivated at least 1 day after confluence)[1] possess a lower CFE and a higher percentage of aborted colonies than keratinocytes cultivated under conditions that privilege colony-forming ability and stem-cell maintenance (keratinocytes passaged during the exponential phase of growth).[21,28] Indeed, the latter culture system is aimed at permanent epidermal regeneration in massive full-thickness burns.[21] To assure that cultured epidermal grafts contained the appropriate number of melanocytes, the M/K was monitored for each patient on parallel secondary cultures seeded in 24-multiwell plates (Figure 1A). As detailed in Table 2, the M/K in cultured grafts ranged between 1:30 and 1:200.

CLINICAL OUTCOME

A total of 2315.8 cm² of body surface were grafted in 21 patients (Table 1). Final evaluation of the percentage of repigmentation was performed after 6 months. As summarized in Table 1, the average percentage of repigmentation was 75.9% (1759.7 cm² repigmented/2315.8 cm²

transplanted). Repigmentation was stable at later follow-up. Although we carefully followed inclusion criteria in selecting patients, patients 19, 20, and 21 developed new lesions 3, 4, and 6 months, respectively, after grafting, suggesting either reactivation of the disease or an erroneous first diagnosis of stable vitiligo. The skin of these patients did not repigment. In contrast, the average percentage of repigmentation in the remaining 18 patients was of 90% (1759.7 cm² repigmented/1953.4 cm² transplanted). No scar formation was observed in any patient (**Figures 2**, **3**, and **4**). As previously reported with different surgical procedures,[1] we did not succeed in inducing repigmentation in upper extremities.

Variation of the melanocyte concentration in cultured grafts within the above range (1:30-1:200) (Table 2) was not correlated with the percentage of final repigmentation, nor with the intensity of repigmentation. In almost all patients, the color of the treated areas was similar to that of the uninvolved surrounding skin. Occasionally (in patients 3 and 5), we observed initial hyperpigmentation that progressively disappeared within the first year after grafting.

COMMENT

Medical treatment of vitiligo aimed at stopping the disease progress and restoring the loss of melanocytes includes the use of corticosteroids, khellin or l-phenylalanine associated with UV-A radiation, psoralens with photochemotherapy (PUVA), and narrowband UV-B.[3,4,7,29-31] Surgical therapy entails melanocyte trans-
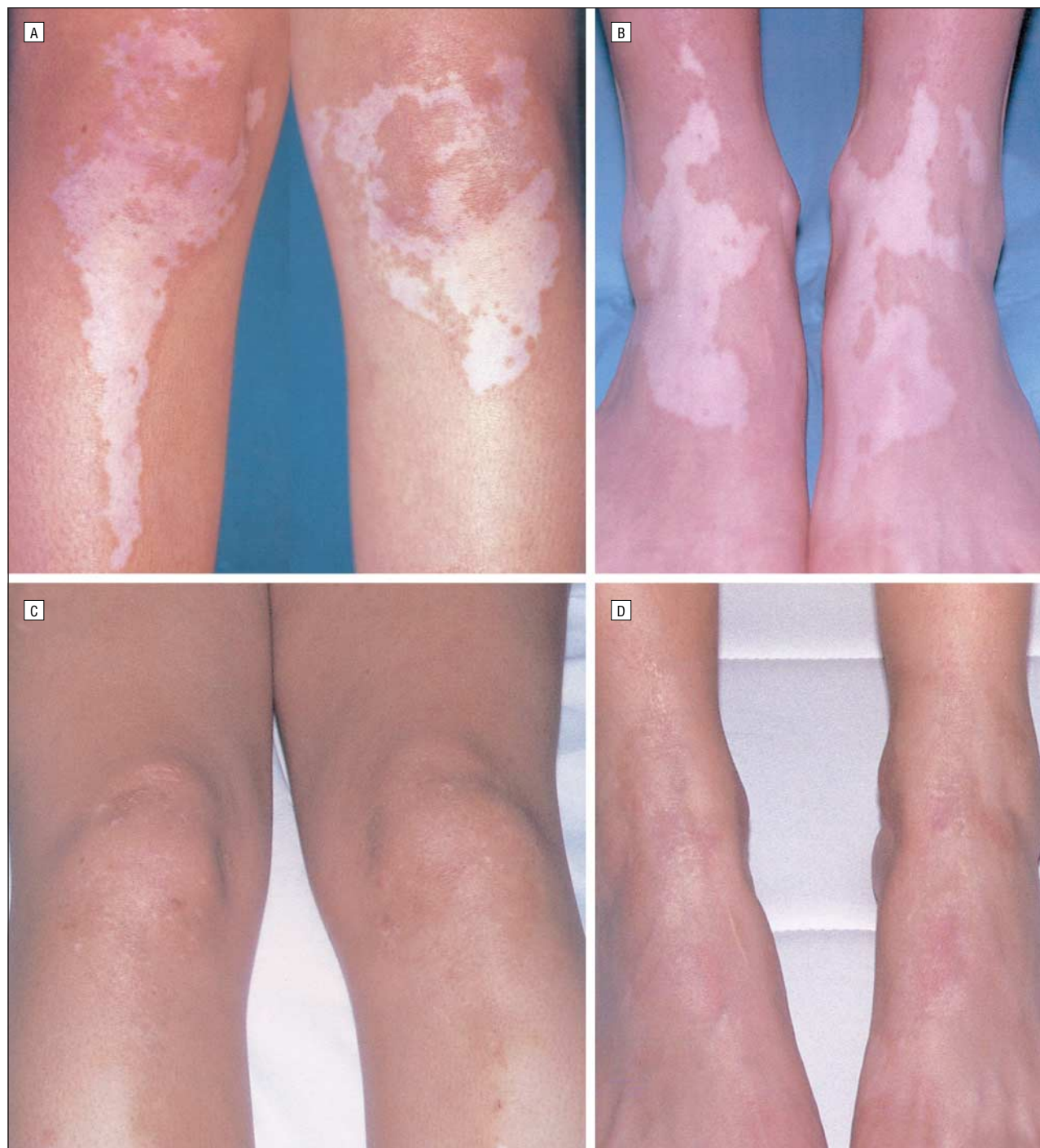
Figure 2. The achromic areas of stable generalized vitiligo on the knees and legs (A) and ankles and feet (B) of patient 3 were treated with 2 serial applications of epidermal cultures. C and D, Complete repigmentation was achieved.

plantation and is demanded when medical therapy fails but only in patients with stabilized disease.[1-4,6-19,32-34]

Several surgical techniques including suction blisters, minigrafts, punch grafts, or split-thickness grafts and flip-top transplants have been proposed for the treatment of stable vitiligo.[2-4,6-19,32-34] Although successful,[2,12-19,32-34] these procedures require multiple donor sites and can produce adverse effects at the donor site (punctiform scars, pigmentary alterations, and/or hypertrophic scarring) as well as at the recipient site (peripheral ipopigmentation,

cobblestone appearance, scar formation, and/or color mismatch).[2,4,6,11]

The main advantage of autologous cultured epidermis is the possibility of producing a large amount of epithelial sheets starting from a small specimen. Obviously, a key issue for the successful clinical outcome of cell therapy deals with the quality controls of the culture system, such as maintaining a proper CFE of confluent keratinocyte cultures and a reproducible range of M/K in confluent cultures. Main disadvantages of using
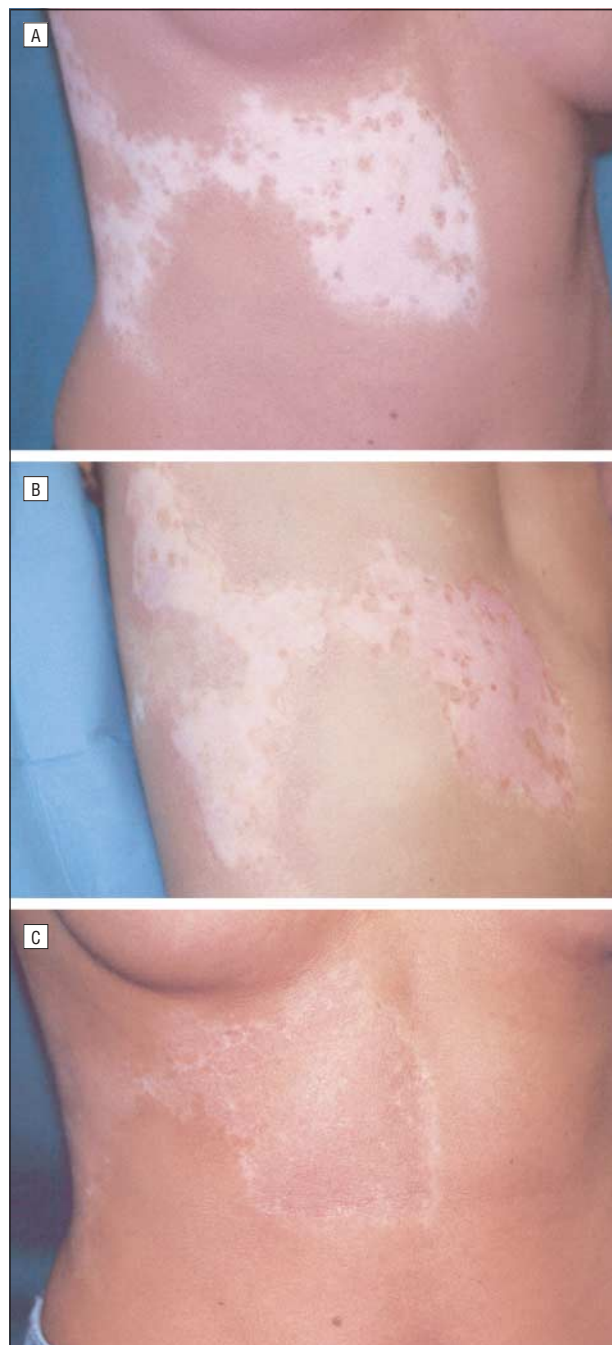
Figure 3. Segmental vitiligo of the abdomen on patient 9 before treatment (A) and after erbium:YAG laser disepithelialization (B). Note the absence of bleeding. C, Almost complete (97%) repigmentation was obtained with a single operation by 6 months after autotransplantation.

Figure 4. A, Pretreatment generalized vitiligo affecting the left region of the pubis and both inguinal folds of patient 10. B, Twelve-month follow-up: a repigmentation of 96% was achieved. C, Achromic hairs were still present 2 months after the autotransplantation. D, Hair repigmentation was observed at 6-month follow-up.

autologous cultured epidermal sheets are certainly the need of safe and controlled cell culture facilities as well as the high level of expertise and number of quality controls required. These factors contribute to the high cost for this type of cell therapy.

We do not sell cultured autografts, so we cannot report a selling price. However, based on the production cost of our cultures, we have calculated a mean cost of approximately $1200 for each 1% of body surface area covered with cultured epidermal sheets. (This estimate includes only the manufacture of the epidermal grafts,
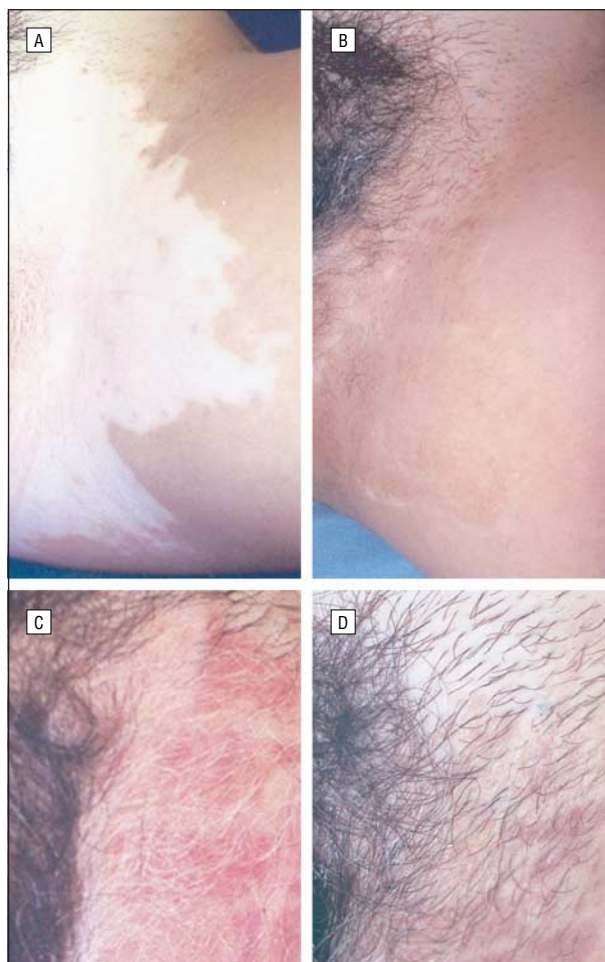
without considering the cost of infrastructures.) It should also be considered, however, that the high number of surgical operations required for standard surgical procedures has an obvious cost, even not considering the loss of patient's time, which impinges on his or her professional activity.

Absence of scar formation can be obtained by the reproducible accuracy of a less invasive disepithelialization procedure. As recently reported, autologous cultured epidermis bearing melanocytes induces repigmentation in stable vitiligo lesions prepared by means of timedsurgery,[1] which allows an optimal preparation of the wound bed and hence a complete repigmentation in the absence of scars.[1] Timedsurgery, however, is a time-consuming procedure. Usually it takes approximately 15 to 20 minutes to remove 50 cm² of epidermis. To reduce the operative time, we used Er:YAG laser surgery that allowed the removal of approximately 50 cm² of epidermis in 7 to 10 minutes. The overall success rate in our study was 75.9% in most of the treated body areas (including feet). This value increases to 90% if we exclude the 3 patients whose vitiligo reactivated and where this technology did not achieve any success.

The choice of the Er:YAG laser was based on the high safety profile of this type of laser. The target of erbium or carbon dioxide laser light is intracellular and extracellular water.[35] Water absorbs the light and vaporizes. The Er:YAG laser emits light within the mid-infrared portion of the electromagnetic spectrum with a wavelength of 2940 nm.[36] This wavelength corresponds to the 3000-nm absorption peak of the water.[36] The absorption coefficient of water for Er:YAG laser light is 12800 cm^{-1}, whereas that for the carbon dioxide laser light is only 800 cm^{-1}, which means that Er:YAG energy is 12 to 18 times more efficiently absorbed by water-containing tissues.[36] Consequently, Er:YAG energy is absorbed more superficially within the skin, leading to precise ablation of the epidermal layer.[26,36] Furthermore, the Er:YAG laser produces a pulse of 250 to 350 microseconds[36]: this pulse is shorter than the thermal relaxation time of the skin (the time it takes for the skin to cool to half of its peak temperature immediately after laser irradiation), which is 1 millisecond.[37,38] Lasers with such short pulses allow a superficial vaporization with a reduced zone of thermal injury.[35,39]

Potential adverse effects of laser surgery are scarring and hyperpigmentation. Although occasional patients can develop scars due to as yet unidentifiable factors,[40] in our opinion, the risk of scarring is mainly related to infection, depth of the wound bed, and/or limited experience of the operator.[40,41] The overall incidence of infections in laser resurfacing is low[40] because laser light sterilizes the wounded area.[39] Although Er:YAG laser disepithelialization has been performed in different ways (that are also related to potential differences between lasers),[12-15] to obtain an adequate depth of the wound bed in the disepithelialization of achromic areas, we ablated slightly less tissue than was necessary to achieve pinpoint bleeding. Pinpoint bleeding indicates penetration into the papillary dermis; therefore, it represents the limit for a safe surgical preparation of the wound bed.[26,38] Finally, transient hyperpigmentation frequently occurs after laser treatment, with a higher frequency in patients with Fitzpatrick III and IV skin types[20,35,36]: in our 2 patients it resolved spontaneously.

Although we chose a long period (18 months) of clinical stability to include patients in this study, vitiligo activity or reactivation occurred in 3 of our 21 patients. There is no consensus regarding the clinical evaluation of disease stability in vitiligo: according to different definitions, it can range from 6 months to 2 years.[42] The minigraft test or test grafting[43,44] has been proposed to detect unstable cases, but there are different opinions on this procedure.[45] The experimentally induced Koebner phenomenon[42] can be a test to assess vitiligo activity, but it can produce false-negative results (unpublished findings, 2001). In fact, among our 3 reactivated cases, depigmentation of the donor site (that could be caused by the skin biopsy as Koebner phenomenon) only occurred in 1 patient. This confirms that tests for an unambiguous definition of vitiligo stability are not easily identifiable.[45] We think that a longer period of disease stability (at least 2 years) should be introduced into our inclusion criteria.

In this study, we were still unable to obtain substantial improvements of achromic lesions of the hands. In anatomic locations such as hands and fingers, diffi-culties in immobilizing cultured grafts can interfere with the graft survival, but other unknown factors could also cause graft failure.[46] After laser disepithelialization on fingers, for example, we saw a greater inflammatory reaction than that observed on other body sites.

Interestingly, and in contrast to what was observed in an earlier study,[1] patient 10 showed repigmented hairs 6 months after grafting. This confirms previous data suggesting that transplanted melanocytes can recolonize the hair bulb by downstream migration from the repigmented epidermis.[47,48] This phenomenon, however, is rare and is controlled by as yet unidentified mechanisms.

In summary, this study confirms that cultured epidermal grafts can achieve high success rates in repigmenting stable vitiligo lesions. A precise surgical technique for epidermal removal and a proper melanocyte concentration within the epidermal grafts can give positive results in a highly reproducible way. Furthermore, removing the epidermis in a fast and safe manner allows treatment of large vitiligo lesions during a single surgical operation.

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REFERENCES

1. Guerra L, Capurro S, Melchi F, et al. Treatment of stable vitiligo by timedsurgery and transplantation of cultured epidermal autografts. *Arch Dermatol*. 2000;136:1380-1389.
2. Njoo MD, Westerhof W, Bos JD, Bossuyt PMM. A systematic review of autologous transplantation methods in vitiligo. *Arch Dermatol*. 1998;134:1543-1549.
3. Njoo MD, Westerhof W, Bos JD, Bossuyt PMM. The development of guidelines for the treatment of vitiligo. *Arch Dermatol*. 1999;135:1514-1521.
4. Njoo MD, Westerhof W. Vitiligo: pathogenesis and treatment. *Am J Clin Dermatol*. 2001;2:167-181.
5. Capurro S, Fiallo P. Epidermal disepithelialization by programmed diathermosurgery. *Dermatol Surg*. 1997;23:600-601.
6. Falabella R. Surgical therapies for vitiligo. *Clin Dermatol*. 1997;15:927-939.
7. Halder RM, Young CM. New and emerging therapies for vitiligo. *Dermatol Clin*. 2000;18:79-89.
8. Falabella R. Surgical therapies for vitiligo and other leukodermas, I: minigrafting and suction epidermal grafting. In: Bolognia JL, Tharp MD, Olsen E, eds. *Dermatologic Therapy: The Treatment of Dyspigmentation*. Malden, Mass: Blackwell Science Inc; 2001:7-14.
9. McGovern TW, Leffell DJ. Surgical therapies, II: flip-top transplants in vitiligo. In: Bolognia JL, Tharp MD, Olsen E, eds. *Dermatologic Therapy: The Treatment of Dyspigmentation*. Malden, Mass: Blackwell Science Inc; 2001:15-19.
10. Leachman SA. Surgical therapies, III: melanocyte transplants. In: Bolognia JL, Tharp MD, Olsen E, eds. *Dermatologic Therapy: The Treatment of Dyspigmentation*. Malden, Mass: Blackwell Science Inc; 2001:20-28.
11. van Geel N, Ongenae K, Naeyaert J-M. Surgical techniques for vitiligo: a review. *Dermatology*. 2001;202:162-166.
12. Yang JS, Kye YC. Treatment of vitiligo with autologous epidermal grafting by means of pulsed erbium:YAG laser. *J Am Acad Dermatol*. 1998;38:280-281.
13. Sachdev M, Shankar DSK. Dermatologic surgery: pulsed erbium:YAG laser-assisted autologous epidermal punch grafting in vitiligo. *Int J Dermatol*. 2000;39:868-871.
14. Sachdev M, Krupashankar DSR. Suction blister grafting for stable vitiligo using pulsed erbium:YAG laser ablation for recipient site. *Int J Dermatol*. 2000;39:471-473.

15. Pai GS, Vinod V, Joshi A. Efficacy of erbium:YAG laser-assisted autologous epidermal grafting in vitiligo. *J Eur Acad Dermatol Venereol.* 2002;16:604-606.
16. Oh C-K, Cha J-H, Lim J-Y, et al. Treatment of vitiligo with suction epidermal grafting by the use of an ultrapulse CO2 laser with a computerized pattern generator. *Dermatol Surg.* 2001;27:565-568.
17. Acikel C, Ulkur E, Celikoz B. Carbon dioxide laser resurfacing and thin skin grafting in the treatment of "stable and recalcitrant" vitiligo. *Plast Reconstr Surg.* 2003; 111:1291-1298.
18. Chen Y-F, Chang JS, Yang P-Y, Hung C-M, Huang M-H, Hu D-N. Transplant of cultured autologous pure melanocytes after laser-abrasion for the treatment of segmental vitiligo. *J Dermatol.* 2000;27:434-439.
19. Chen Y-F, Yang P-Y, Hung C-M, Hu D-N. Transplantation of autologous cultured melanocytes for treatment of large segmental vitiligo. *J Am Acad Dermatol.* 2001; 44:543-544.
20. Sapijaszko MJA, Zachary CB. Er:YAG laser skin resurfacing. *Dermatol Clin.* 2002; 20:87-96.
21. Pellegrini G, Ranno R, Stracuzzi G, et al. The control of epidermal stem cells (holoclones) in the treatment of massive full-thickness burns with autologous keratinocytes cultured on fibrin. *Transplantation.* 1999;68:868-879.
22. Barrandon Y, Green H. Three clonal types of keratinocytes with different capacities for multiplication. *Proc Natl Acad Sci U S A.* 1987;84:2302-2306.
23. De Luca M, D'Anna F, Bondanza S, Franzi AT, Cancedda R. Human epithelial cells induce human melanocyte growth in vitro but only skin keratinocytes regulate its proper differentiation in the absence of dermis. *J Cell Biol.* 1988;107:1919-1926.
24. De Luca M, Franzi AT, D'Anna F, et al. Coculture of human keratinocytes and melanocytes: differentiated melanocytes are physiologically organized in the basal layer of the cultured epithelium. *Eur J Cell Biol.* 1988;46:176-180.
25. Stratigos AJ, Dover JS. Overview of lasers and their properties. In: Alster TS, Tharp MD, Olsen E, Phillips J, eds. *Dermatologic Therapy: The Use of Lasers in Dermatology.* Malden, Mass: Blackwell Science Inc; 2000:2-16.
26. Weinstein C. Erbium laser resurfacing: current concepts. *Plast Reconstr Surg.* 1999;103:602-616.
27. Kaufmann R. Role of erbium:YAG laser in the treatment of aged skin. *Clin Exp Dermatol.* 2001;26:631-636.
28. Rama P, Bonini S, Lambiase A, et al. Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. *Transplantation.* 2001;72:1478-1485.
29. Njoo MD, Spuls PI, Bos JD, et al. Nonsurgical repigmentation therapies in vitiligo: meta-analysis of the literature. *Arch Dermatol.* 1998;134:1532-1540.
30. Halder RM, Brooks HL. Medical therapies for vitiligo. In: Bolognia JL, Tharp MD, Olsen E, eds. *Dermatologic Therapy: The Treatment of Dyspigmentation.* Malden, Mass: Blackwell Science Inc; 2001:1-6.
31. Taneja A. Treatment of vitiligo. *J Dermatolog Treat.* 2002;13:19-25.
32. Mutalik S, Ginzburg A. Surgical management of stable vitiligo: a review with personal experience. *Dermatol Surg.* 2000;26:248-254.
33. Yaar M, Gilchrest BA. The evolution of cultured epidermal autografts and other surgical treatment modalities. *Arch Dermatol.* 2001;137:348-349.
34. Olsson MJ, Juhlin L. Long-term follow-up of leucoderma patients treated with transplants of autologous cultured melanocytes, ultrathin epidermal sheets and basal cell layer suspension. *Br J Dermatol.* 2002;147:893-904.
35. Ratner D, Tse Y, Marchell N, Goldman MP, Fitzpatrick RE, Fader DJ. Cutaneous laser resurfacing. *J Am Acad Dermatol.* 1999;41:365-389.
36. Alster TS, Lupton JR. Erbium:YAG cutaneous laser resurfacing. *Dermatol Clin.* 2001;19:453-466.
37. Perez MI, Bank DE, Silvers D. Skin resurfacing of the face with the erbium:YAG laser. *Dermatol Surg.* 1998;24:653-659.
38. Alster TS. Cutaneous resurfacing with CO2 and erbium:YAG lasers: preoperative, intraoperative, and postoperative considerations. *Plast Reconstr Surg.* 1999; 103:619-632.
39. Hruza GJ. Laser treatment of epidermal and dermal lesions. *Dermatol Clin.* 2002; 20:147-164.
40. Fitzpatrick RE. CO2 and Er:YAG laser resurfacing: practical approaches. In: Alster TS, Tharp MD, Olsen E, Phillips J, eds. *Dermatologic Therapy: The Use of Lasers in Dermatology.* Malden, Mass: Blackwell Science Inc; 2000:102-113.
41. McBurney EI. Side effects and complications of laser therapy. *Dermatol Clin.* 2002; 20:165-176.
42. Njoo MD, Das PK, Bos JD, Westerhof W. Association of the Köbner phenomenon with disease activity and therapeutic responsiveness in vitiligo vulgaris. *Arch Dermatol.* 1999;135:407-413.
43. Falabella R, Arrunategui A, Barona MI, Alzate A. The minigrafting test for vitiligo: detection of stable lesions for melanocyte transplantation. *J Am Acad Dermatol.* 1995;32:228-232.
44. Malakar S, Dhar S. Treatment of stable and recalcitrant vitiligo by autologous miniature punch grafting: a prospective study of 1,000 patients. *Dermatology.* 1999;198:133-139.
45. Malakar S. How unstable is the concept of stability in surgical repigmentation of vitiligo? *Dermatology.* 2000;201:182-183.
46. Ongenae K, van Geel N, Naeyaert JM. Autologous cellular suspensions and sheets in the treatment of achromic disorders: the need for future controlled studies. *Dermatology.* 2001;202:158-161.
47. Falabella R, Escobar C, Borrero I. Treatment of refractory and stable vitiligo by transplantation of in vitro cultured epidermal autografts bearing melanocytes. *J Am Acad Dermatol.* 1992;26:230-236.
48. Kumagai N, Uchikoshi T. Treatment of extensive hypomelanosis with autologous cultured epithelium. *Ann Plast Surg.* 1997;39:68-73.