Fátima Regina Vilani-Moreno[1]
Heloísa Cristina Quatrini Carvalho Passos Guimarães[2]
Sidinéia Raquel Bazalia Bassoli[3]
Roseli Marega Oda[4]
Érika Mozer[5]
Wladimir Fiori Bonilha Delanina[6]
Maria Ângela Bianconcini Trindade[7]
Joel Carlos Lastória[8]
Norma Gondim Cleto[9]

IMPLANTATION OF AUTOLOGOUS KERATINOCYTES IN LOWER LIMB ULCERS OF PATIENTS WITH LEPROSY SEQUELAE

ABSTRACT

The occurrence of ulcers in leprosy is mainly due to the existence of numb areas that can easily undergo trauma and burns and thereafter become secondarily infected. This study aimed to treat ulcers in lower limbs of patients with leprosy sequelae through the implantation of autologous keratinocytes. The study consisted of fourteen patients (six with a single ulcer, four with more than one ulcer in the same limb and four with ulcers in both limbs) with a total of 31 ulcers. A fragment of healthy skin was collected and subjected to enzymatic digestion to obtain keratinocytes, which were then cultured for four weeks. The resulting keratinocytes were implanted in ulcers in combination with fibrin glue. Patients were followed for two months and the ulcers were monitored weekly during this time. Ulcers measuring ≤ 9.0 cm high x 5.0 cm wide (23 ulcers) decreased by 68.8% ± 27.1%, with nine of them showing complete healing. Larger ulcers decreased by 50.0% ± 31.9%, with two of these healing completely. Thus, implantation of autologous keratinocytes in combination with fibrin glue was effective in healing and/or reducing the ulcer size and should be considered as an additional ulcer treatment option in patients with leprosy sequelae.

Key words: leprosy, keratinocytes, lower limb ulcers, fibrin glue.

Vilani-Moreno FR, Guimarães HCP, Bassoli SRB, Oda RM, Mozer E, Delanina WFB, Trindade MAB, Lastória JC, Cléto NG. Implantation of autologous keratinocytes in lower limb ulcers of patients with leprosy sequelae. Hansen Int 2012; 37(1): 19-24.

INTRODUCTION

Regardless of their etiology, skin ulcers, particularly those of lower limbs, cause great suffering and compromise the quality of life of patients and their families.[1] Lower limbs are subject to various types of ulcers due to their frequent exposure to trauma and less efficient circulation caused by the nature of the human stance.

Received: 18 june 2012
Accepted: 01 september 2012
Published online: 10/08/2013

1 PhD, Scientific Researcher, Equipe Técnica de Biologia, Instituto Lauro de Souza Lima, Bauru, SP, Brazil. fmoreno@ilsl.br
2 PhD, Scientific Researcher, Equipe Técnica de Clínica e Terapêutica, Instituto Lauro de Souza Lima, Bauru, SP, Brazil. clinicapes@ilsl.br
3 MSc, Nurse, Divisão de Enfermagem, Instituto Lauro de Souza Lima, Bauru, SP, Brazil. silvissoli@ig.com.br
4 MSc, Nurse, Divisão de Enfermagem, Instituto Lauro de Souza Lima, Bauru, SP, Brazil. enfermagem@ilsl.br
5 BS, Biology, Equipe Técnica de Imunologia, Instituto Lauro de Souza Lima, Bauru, SP, Brazil. emozermozer@yahoo.com.br
6 MD, Divisão de Dermatologia, Instituto Lauro de Souza Lima, Bauru, SP, Brazil. wladelanina@uol.com.br
7 MD, PhD, Scientific Researcher, Instituto de Saúde e Divisão de Clínica Dermatológica, Hospital das Clínicas, Faculdade de Medicina/USP, São Paulo, SP, Brazil. angelatrindade@uol.com.br
8 MD, PhD, Professor, Departamento de Dermatologia, Faculdade de Medicina de Botucatu, UNESP, Botucatu, SP, Brazil. lastoria@fmb.unesp.br
9 MD, Divisão de Dermatologia, Instituto Lauro de Souza Lima, Bauru, SP, Brazil. normagc@uol.com.br
Correspondence to: Fátima Regina Vilani Moreno, Instituto Lauro de Souza Lima, Rod. Cmte. João Ribeiro de Barros, Km 225/226, 17034-971 Bauru, SP, Brazil. Phone: +55 14 31035912 Fax: +55 14 31035914. E-mail: fmoreno@ilsl.br
Financial support: CNPq and Fundação Paulista contra a Hanseníase

With leprosy, ulcers can arise due to numb areas that can easily be traumatized, burned and secondarily infected.[2]

According to De Las Aguas,[3] around 1.8 million leprosy patients have physical disabilities and many of these have recurring ulcers in the lower limbs. In the lepromatous form, these ulcers are distinct because skin in the patients' legs is infiltrated with the possible presence of papules, nodules and leprosy lesions. Both the diffuse infiltrations and the leprosy lesions may ulcerate due to the blockage of blood vessels by inflammatory infiltrate that includes large amounts of macrophages containing bacilli. In advanced cases of this form of leprosy, the superficial veins can be completely blocked by the lepromatous infiltration, which can cause pan phlebitis. In addition, there are often neurological alterations in the lower limbs, leading to impaired sudoresis and altered circulation in small dermal vessels, which contributes to the onset of ulcers.[2]

Surgical treatment of the ulcers includes auto grafts, but is limited by its requirement for large areas of skin from the donor area. Technological advances have made it possible to cultivate unlimited quantities of replacement skin from a small skin sample *in vitro*.[4-6] Using these techniques, researchers have successfully used keratinocytes from culture for restoring skin lesions.[4, 7, 8] These cells can be grown on a substrate, such as acellular human dermis or a transparent film, or be isolated in culture and then implanted on ulcers with fibrin glue.[7, 9, 10]

Because ulcer development in leprosy patients is a complex process, their treatment should be based on the identification and elimination of the various contributing factors. Although there are many topical substances, sophisticated covers and several surgical techniques, treatment should be multidisciplinary and individualized. Therefore, this study aimed to heal lower limb ulcers of patients with leprosy sequelae using an alternative treatment.

MATERIALS AND METHODS

Patients: fourteen patients with chronic lower limb ulcers from leprosy sequelae treated at the outpatient clinic of the Instituto Lauro de Souza Lima, Bauru, Brazil, during the period of March 2007 to February 2009 participated in this study. Of the fourteen patients, ten (71%) were male, aged 28-74 years (mean 51 years), and four (29%) were female, aged 55-75 years (mean 63 years). All patients had completed treatment specifically for leprosy and had sequelae of the disease. Of these, nine had the lepromatous clinical form of the disease and five were borderline. The average evolution time of the ulcers was 10.5 years (10 years for males and 11 years for females). Of the fourteen patients evaluated, six had a single ulcer, four had more than one ulcer in the same leg and four had ulcers in both legs, for a total of 31 ulcers. The inclusion criterion was defined as no arterial compromise in the lower limb that contained the ulcer. Before receiving the implant, the ulcers were evaluated and prepared, such that they were uninfected and free of devitalized tissues. This study was approved by Ethics Committee of the Instituto Lauro de Souza Lima and only included willing patients who signed a consent form.

Collection and preparation of skin samples for culture: a piece of healthy skin from the abdominal region was obtained from every patient and subjected to enzymatic digestion to obtain keratinocytes. Briefly, the samples were washed in 0.9% saline solution containing an antibiotic-antimycotic mixture (100 IU/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml amphotericin - Gibco, Grand Island, NY, USA). After removal of adipose tissue, the dermis/epidermis was cut into small 1-2 mm pieces. The fragments were placed in a Petri dish with trypsin-EDTA (Gibco) and incubated at 37ºC for 4 h in an atmosphere of 5% CO_2. Trypsin was neutralized by the addition of fetal calf serum (Gibco). The supernatant was collected, filtered through a 40-µm nylon filter (BD Falcon, Bedford, MA, USA) and then centrifuged at 200 g for 10 min. The cells were washed once with 0.9% saline solution and the cell pellet was resuspended in saline. Cell counting was performed using a 1:2 dilution with 5% Turck dye in 4% glacial acetic acid. Cell viability was determined by dilution of cells 1:2 in 0.1% trypan blue dye in buffered saline, pH 7.2.

Keratinocytes culture: the cell concentration was adjusted to 1 x 10^6/ml using keratinocyte culture media (Keratinocyte-SFM, Gibco) supplemented with 2 mM/ml L-glutamine, 5 ng/ml epidermal growth factor, 50 µg/ml bovine pituitary extract, 10% fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were plated in 25-cm^2 cell culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated at 37ºC in a 5% CO_2 atmosphere. After cells had adhered to the flasks, the medium was removed and replaced every two days. Cells were removed from the culture flasks for use in the grafts of patient ulcers when the cultures were confluent, which was approximately after 4 weeks in culture.

Post-culture keratinocyte recovery: after removal of the culture medium, trypsin-EDTA was added to the flask. The flask was incubated at 37ºC in a 5% CO_2 atmosphere for 5 min. Trypsin was neutralized with fetal calf serum. The keratinocytes were harvested, centrifuged and washed once with 0.9% saline solution. Cell pellets were resuspended in 1 ml thrombin (one of the components of the fibrin glue kit), and the total cell number and viability were determined. After reconstitution in fibrinogen from the fibrin glue kit, the application kit (Conjunct Pantaject®) was ready to be applied to the patient's ulcer.

Keratinocyte implantation in ulcers: before receiving the cell implant, patient ulcers were photographed and evaluated for their appearance and size. Cells were grafted into the ulcer together with fibrin glue (Beriplast®-P, ZLB Behring GmbH, Marburg, Germany). The ulcer was then covered with Adaptic (Johnson & Johnson), a non-adherent dressing, and an Unna boot (ConvaTec, Bristol-Myers Squibb, NY, USA), as described by Phillips *et al.*[11] Rest for 24 h was recommended to the patients.

Patient monitoring: ulcers were washed weekly with 0.9% physiological serum. They were measured using a sterile paper ruler and photographed. Ulcers were covered with Adaptic dressing and an Unna boot. Patients were followed for two months. The implantation of cells was repeated in three patients with extensive ulceration who agreed to the new implant. In these cases, the same procedures described above were used and the ulcer was evaluated for another 2 months, as described.

RESULTS

The results obtained from the keratinocyte implantation are summarized in Table 1. Ulcers measuring ≤ 9.0 cm high x 5.0 cm wide (23 ulcers) showed a 68.8% ± 27.1% (median 64%) reduction in size. Nine of these ulcers healed completely. Ulcers larger than these showed a 50.0% ± 31.9% (median 36%) reduction in size, and two healed completely. Figure 1 shows pictures of one patient's ulcer before and two months after implantation.

Table 1 Ulcer evolution two months after keratinocyte implantation in patients with leprosy sequelae.

Patient number/ clinical form	Age (years)	Location/ number of ulcers	Initial measurement (height x width) (cm)	Final measurement (height x width) (cm)	% Reduction
01/LL	74	LIL/02	6.0 x 4.0 13.5 x 7.5	2.0 x 1.5 9.0 x 4.5	65 37
02/LL	60	RIL/01	5.0 x 3.0	healed	100
03/BL*	56	LIL/01	21.0 (circular and irregular)	16.0	24
04/L	55	RIL/01	11.0 x 4.5	healed	100
05/BL	28	LIL/01 RIL/01	7.5 x 2.0 14.5 x 10.0	healed 7.4 x 5.0	100 50
06/BL	45	LIL/02	1.5 x 2.0 4.5 x 2.0	1.0 x 0.8 3.0 x 1.0	47 42
07/LL	65	RIL/01	2.0 x 4.0	1.0 x 3.0	38
08/BL	48	LIL/01	10.3 x 7.3	6.5 x 6.0	28
09/LL	75	LIL/01	1.6 x 2.2	1.0 x 0.9	48
10/BL	56	LIL/03	2.0 x 2.0 12.0 x 2.5 5.0 x 8.0	healed healed 2.5 x 1.8	100 100 64
11/LL*	42	LIL/03	3.5 x 0.5 4.0 x 5.0 5.0 x 5.0	healed 1.0 x 2.0 2.0 x 2.0	100 68 60
		RIL/01	16.0 cm (circular)	10.5	35
12/LL	62	LIL/04	2.0 x 2.5 3.5 x 1.0 1.5 x 0.8 3.0 x 1.4	healed healed healed healed	100 100 100 100
		RIL/02	8.5 x 5.0 5.5 x 5.0	2.4 x 3.8 3.5 x 1.8	50 50
13/LL*	40	LIL/02	2.0 x 3.5 3.0 x 3.0	healed 2.0 x 2.0	100 33
		RIL/01	13.5 cm (circular)	10.0	26
14/LL	60	RIL/03	6.0 x 2.0 5.5 x 4.0 6.2 x 6.0	3.5 x 1.0 2.8 x 3.0 5.0 x 3.0	46 37 35

LL= Lepromatous Leprosy; BL= Borderline Leprosy; RIL= Right Inferior Limb; LIL= Left Inferior Limb.

* patient subjected to two cell implants.

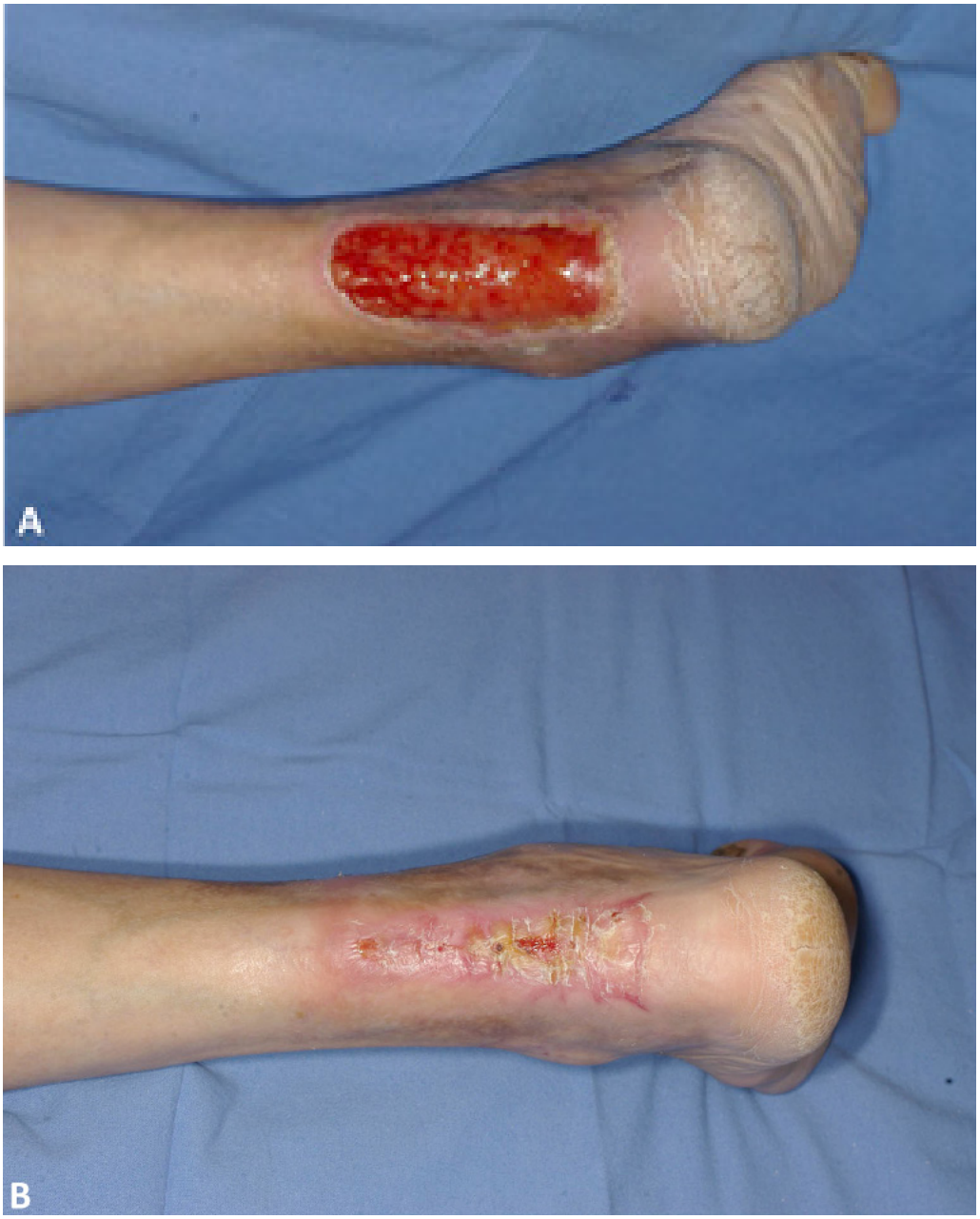

Figure 1 Ulcer in lower limb of patient with leprosy sequelae subjected to keratinocyte implantation. **A.** Before implant. **B.** Two months after implant – 100% healed.

The medium ulcer size before implantation was 6.0 x 4.2 cm in males and 4.9 x 3.1 cm in females, not including three circumferential and irregular ulcers that were treated. Two months post-implantation, the medium ulcer size was 1.8 x 1.7 cm in males and 1.7 x 1.2 cm in females, which are 65% and 63% reductions in size, respectively.

The correlation between patient age and evolution of the ulcers after 2 months of implantation revealed that complete healing occurred in 11 patients aged 28-68 years (Table 2). In regards to the evolution of the ulcers pre and post- implantation, we found that of the eleven healed ulcers, five occurred in patients who had ulcers for up to five years, two in patients with ulcers for up to ten years and four in patients with ulcers for over nineteen years (Table 3).

Table 2 Correlation between patient age and evolution of ulcers after cell implantation in patients with leprosy sequelae.

	Number of Ulcers		
Age (years)	**Healed**	**Reduced - % reduction**	
28-48	3	9	43.2
49-68	8	8	43.0
> 70	0	3	50.0

Table 3 Correlation between ulcer evolution time before cell implantation and two months after procedure in patients with leprosy sequelae.

Time of evolution (years)	Number of Ulcers		
	Healed	Reduced - % reduction	
3-5	5	3	54.0
8-10	2	9	41.7
> 19	4	8	43.1

Patients that received two implants had extensive and irregular ulcers, which covered almost the entire circumference of the leg. In these patients, we observed a 28% reduction in the size of the ulcers.

DISCUSSION

A large number of people with leprosy, in most cases already treated, can develop ulcers in their legs as sequelae of the disease. These ulcers are chronic and characterized by long healing times. We conducted this study to develop an alternative method to effectively treat and heal lower limb ulcers. In this study, most patients (64%) who underwent cell implantation had the lepromatous clinical form of leprosy. Previous studies by Siddiqui *et al.*[12] and Salazar *et al.*[13] also showed that ulcers are predominant (54.7% and 95.4%, respectively) in patients with this form of the disease. These findings are possibly due to the fact that these patients show diffuse infiltrations that ulcerate due to blockage of blood vessels by the inflammatory infiltrate, which has large amounts of bacilli-containing macrophages, as well as the presence of neural involvement in more advanced forms of the disease.[2, 3]

Of the patients evaluated, 21% had other associated systemic conditions, such as hypertension and *diabetes mellitus*, which could interfere with the healing process. These patients showed a 49% reduction in their ulcers and five completely healed. These findings are not in agreement with those reported by Nelzén *et al.*,[14] who observed worsening of lower limb ulcers in patients suffering from leprosy and related diseases.

Previous studies suggest that advanced age may hinder healing and contribute to the worsening of ulcers.[15, 16] In this study, the patients' ages ranged from 28 to 75 years and the ulcer size reduction rate was similar in all age groups. The number of healed ulcers was actually greater in the age group of 49-68 years. Our results are similar to those of Trier *et al.*,[17] who found no statistical difference among patients of different etiologies and age groups undergoing grafts. In a similar study, Ceilley *et al.*[18] also found no correlation between advanced age and decrease in wound diameter after grafting. Phillips *et al.*[11] previously reported that keratinocytes of individuals over 60 years cultured *in vitro* grow more slowly and cannot achieve confluency in culture. Furthermore, they showed that the survival span of these cells is shorter and that the response to mitogens is less robust, thus suggesting that these characteristics tend to persist after grafting in wounds. Our study included four patients aged over 60 years, one of whom showed complete healing of four ulcers and the remaining three had a 48% reduction in ulcer size. In cultures of keratinocytes, we found no differences in relation to cultures of patients aged less than 60 years.

Implantation of skin cells has been used with success for the treatment of ulcers of various etiologies.[7, 11, 19] To date, there are no reports in the literature describing the use of *in vitro* cultured autologous skin cells for the treatment of chronic ulcers of patients with sequelae of leprosy. In this study, we implanted these cells after cultivation and dilution in fibrin glue using a commercial kit according Siedler & Schüller-Petrovic[9] and Puzzi *et al.*[10] These authors reported success in treating arteriovenous ulcers and ulcers from sickle cell anemia patients using cells resuspended in this vehicle.

Fibrin glue is composed of fibrinogen and factor XIII reconstituted in aprotinin solution, an antifibrinolytic agent. The other component of the glue is thrombin reconstituted in calcium chloride, which leads to rapid solidification of the glue. In the final stage of coagulation, fibrinogen and thrombin form a fibrin clot, which is stable in the presence of factor XIII and calcium.[9]

The healing process is a complex biological event involving cell migration and proliferation, angiogenesis, matrix remodeling and re-epithelialization, with several growth factors and cytokines participating in the process.[19, 20] Several of the involved growth factors are epidermal grown factor (EGF), fibroblast growth factor (FGF), keratinocyte growth factor (KGF) and platelet-derived growth factor (PDGF), which all stimulate cell proliferation and differentiation.[21] In addition, pro-inflammatory cytokines, such as interleukin 1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α), stimulate the expression of KGF and the proliferation of keratinocytes and fibroblasts. In addition, the presence of transforming growth factor-beta (TGF-β) is also crucial for the healing process as it induces angiogenesis, is chemotactic for fibroblasts and stimulates the synthesis of collagen and fibronectin.[19] We did not evaluate the participation of cytokines and growth factors in the patients' ulcers before and after the implant in this study. It is possible that the differences in ulcer size reduction or healing among the patients may be related to these factors and should be studied further.

Several studies suggest that the evolution time of ulcers can be a factor hindering healing because chronic ulcers can be characterized by high concentrations of proteolytic enzymes, a prevalence of older fibroblasts with low replication capacity and a deficiency in the recruitment of keratinocytes to cover the wound bed.[19,

[22, 23] Analysis of individual results in this study suggests that the time of ulcer evolution did not seem to influence their size reduction or healing. These results are similar to those reported by Kunst[15] who also did not find any significant correlation between the reduction of ulcer diameter and its time of evolution in patients with sequelae of leprosy.

In this study, three patients with extensive and circular ulcers were subjected to two cell implants that resulted in a 28% reduction of the ulcer size. We concluded that new implants are necessary in cases such as these, with large ulcers, as previously described by Villeneuve *et al*.[7] These authors reported the healing of an ulcer of 32 cm^2 from a patient with rheumatoid arthritis after four cell implants.

We found complete healing of 35.5% of the ulcers treated and a size reduction of up to 50% in 22.5% of them, and we conclude that implantation of autologous keratinocytes in combination with fibrin glue is effective in healing and/or reducing the size of ulcers and constitutes an additional treatment option for lower limb ulcers of patients with leprosy sequelae.

ACKNOWLEDGMENTS

We thank Coordenação Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Paulista Contra a Hanseníase for financial support.

REFERENCES

1 Phillips T, Stanton B, Provan A. A study of impact of leg ulcers on quality of life: financial, social and psychology implications. J Am Acad Dermatol 1994; 31: 49-53.

2 Opromolla DVA. Úlceras da Perna. In: Jorge AS, Dantas SRPE. Abordagem Multiprofissional do Tratamento de Feridas. São Paulo: Editora Atheneu; 2003. p. 271-8.

3 De Las Aguas JT. Ulceraciones en la lepra. Tratamiento. Leprologia 2001; 4: 248-56.

4 Valvi-Nagy IT, Murphy GF, Mancianti ML, Whitaker D, Herlyn M. Phenotypes and interactions of human melanocytes and keratinocytes in an epidermal reconstruction model. Lab Invest 1990; 62: 314-24.

5 Regnier M, Patwardhan A, Scheynius A. Reconstructed human epidermis composed of keratinocytes, melanocytes and Langerhans cells. Med Biol Eng Comput 1998; 36: 821-4.

6 Carsin H, Ainaud P, Le Bever H, Rives J, Lakhel A, Stephanazzi J, *et al.* Cultured epithelial autografts in extensive burn coverage of severely traumatized patients: a five year single-center experience with 30 patients. Burns 2000; 26: 379-87.

7 Villeneuve P, Hafner J, Prenosil JE, Elsner P, Burg G. A novel culturing and grafting system for the treatment of leg ulcers. Br J Dermatol 1998; 138: 849-51.

8 Terskikh VV, Vasiliev AV. Cultivation and transplantation of epidermal keratinocytes. Int Rev Cytol 1999; 188**:** 41-72.

9 Siedler S, Schüller-Petrovic S. Allogenic keratinocytes suspended in human fibrin glue used for wound healing support in chronic leg ulcers. Arch Dermatol 2000; 136: 676-8.

10 Puzzi MB, Rehder J, Souto LRM, Santos MAG. Implant of autologous fibroblast and keratinocytes in skin ulcers of patients with sickle cell anaemia. In: 4º International Symposium; Reims; 2002.

11 Phillips TJ, Kehinde O, Green H, Gilchrest BA. Treatment of skin ulcers with cultured epidermal allografts. J Am Acad Dermatol 1989; 21: 191-9.

12 Siddiqui MR, Moreira AL, Negesse Y, Taye GA, Hanekon WA, Haslet PAJ, *et al.* Local nerve in damage leprosy does not lead to an impaired cellular immune response or decreased wound healing in the skin. J Infect Dis 2002; 186: 260-5.

13 Salazar JJ, Serrano GG, Leon-Quintero GI, Torres-Mendonza BM. Use of topical ketaserin for treatment of ulcers in leprosy patients. Indian J Lepr 2001; 73: 103-10.

14 Nelzén O, Bergqvist D, Lindhagen A. Venous and non venous leg ulcers: clinical history and appearence in a population study. Br J Surg 1994; 81: 182-7.

15 Kunst H. Predisposing factors for recurrent skin ulcers in leprosy. Lepr Rev 2000; 71: 363-8.

16 Cardia COC. Use of skin graft "punch graft" type for the healing of leg ulcers in treated Hansen's disease patients. J Venom Anim Toxins incl Trop Dis 2006; 12: 316.

17 Trier WC, Peacock EE, Madden JW. Studies on the effectiveness of surgical management of chronic leg ulcers. Plast Reconstr Surg 1970; 45: 20-3.

18 Ceilley RI, Rinek MA, Zuehlke RL. Pinch grafting for chronic ulcers on lower extremities. J Dermatol Surg Oncol 1977; 3: 303-9.

19 Limat A, French LE, Blal L, Saurat JH, Hunziker T, Salomon D. Organotypic cultures of autologous hair follicle keratinocytes for the treatment of recurrent leg ulcers. J Am Acad Dermatol 2003; 48: 207-14.

20 Li J, Chen J, Kirsner R. Pathophysiology of acute wound healing. Clin Dermatol 2007; 5: 9-18.

21 Rothe M, Falanga V. Growth factors. Their biology and promise in dermatologic disease and tissue repair. Arch Dermatol 1989; 125: 1390-8.

22 Yager DR, Nwomeh BC. The proteolytic environment of chronic wounds. Wound Rep Reg 1999; 7: 433-41.

23 Cavallini M. Autologous fibroblasts to treat deep and complicated leg ulcers in diabetic patients. Wound Rep Reg 2007; 15**:** 35-8.