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Clinical results of an autologous engineered skin

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Abstract

Introduction: An artificial complete skin (dermis and epidermis) model has been developed in the Tissue engineering unit of the Centro Comunitario de Sangre y Tejidos del Principado de Asturias (CCST) and CIEMAT. This engineered skin has been employed for the treatment of severe epithelial injuries. In this paper, the clinical results obtained with this engineered skin during the last 18 months were evaluated. *Patients, material and methods*: (a) Culture: Cells (fibroblasts and keratinocytes) were obtained from biopsies by a double enzymatic digestion. After an expansion period, fibroblasts were seeded in an artificial dermis based on human plasma. Keratinocytes were seeded over this dermal surface. (b) Patients: 20 skin biopsies were processed (13 burned patients, 5 giant nevus, 1 GVHD, 1 neurofibromatosis), which came from different hospitals across the country. About 97,525 cm^2 of engineered skin were cultured. *Results*: The engineered skin took in all patients. The take percentage depended on previous pathology (burned patients 10–90%; non-critical patients 70–90%). The epithelization obtained was permanent in all cases. During the follow-up period, epithelial loss, blistering injuries or skin retractions were not observed. The aesthetic and functional results were acceptable. *Conclusions*: This artificial skin has demonstrated to be useful for the definitive treatment of patients with severe skin injuries. This work shows that it is possible to produce this prototype in an hospitalarian laboratory and distribute it to different hospitals across the country.

Introduction

Skin tissue engineering techniques have improved the treatment of severe skin injuries. However, their high cost and scarce availability in Spain have limited their use.

According to the Spanish law, cultured keratinocytes and other products of tissue engineering with living human cells are considered human tissues and their production and distribution comes into the tissue bank activity. In 1994 our bank (Centro Comunitario de Sangre y Tejidos del Principado de Asturias, CCST) started a skin tissue engineering programme for the treatment of severely burned patients. At the beginning, Green's culture technique was employed (Rheinwald and

Green 1975). This method is based on the culture of epithelial cells with lethally irradiated 3T3 cells. Later, due to the problems this technique presented (excessive fragility, loss of cultured surface with the dispase II treatment and problems in the transport of keratinocyte sheets to hospitals located far away from our bank), our bank and CIEMAT started a research programme to develop artificial dermal components for our keratinocyte culture. In this field, among the many possibilities of dermal scaffold described (Bell et al. 1981, 1983; Cooper et al. 1991, 1993; Otto et al. 1995; Rennekampff et al. 1996) we have chosen those based on fibrin, due to the high expansion rate this material has shown (Meana et al. 1998; Pellegrini et al. 1999) for the keratinocyte culturing. Finally, a new prototype of artificial skin based on human plasma as a dermal scaffold has been developed (Llames et al. 2004).

In this paper we describe the culture technique, the clinical activity and results obtained with this skin prototype in the last 18 months.

Patients, material and methods

Skin biopsy collection

When a hospital requests a skin culture, a biopsy collection kit is sent. This kit contains one insulated container, two refrigerant bags, a 100 ml vial of RPMI for the collection of the biopsy, supplemented with antibiotic-antimycotic solution 2% (Life Technologies, final concentration: penicillin 200 U/ml, streptomicin 200 μg/ml and amphotericin 0.5 μg/ml), another small container to introduce the vial to protect the sample and instructions for the collection. All this, along with the patient's data, is sent to our laboratory by express courier.

Obtaining cells for culture

The biopsy was minced using surgical scissors with no previous dermal–epidermal separation. The fragments obtained were enzymatically digested with trypsin (0.05%)/EDTA (0.02%) (T/E). Every 30 min T/E was changed for a fresh T/E mixture. The removed T/E was inactivated with serum-containing culture medium (DMEM + 10% FCS) and centrifuged at $400 \times g$ for 10 min. The pellet was resuspended in culture medium and cells were counted using a Bright-Line® Hemacytometer (Sigma). The procedure was repeated until no more cells were obtained from the sample. The T/E solution was then completely eliminated and the remaining skin biopsy was introduced in a collagenase type I solution (2 mg/ml) (Sigma), containing antibiotic-antimycotic solution 2% (Life Technologies, final concentration: penicillin 200 U/ml, streptomicin 200 μg/ml and amphotericin 0.5 μg/ml) until its complete disaggregation (between 8 and 12 h). The collagenase solution was filtered through a 40 μm filter (Falcon, Becton-Dickinson) and centrifuged at $400 \times g$ for 10 min. The pellet was resuspended in culture medium and cells were counted (Figure 1).

Primary keratinocyte culture

Cells obtained after T/E digestion were seeded in a 75 cm^2 culture flask (2×10^6 cells/flask) in the presence of 8×10^6 lethally (6000 rads) irradiated 3T3 cells (European collection of animal cell culture, no. 85022108) and cultured following the method initially described by Rheinwald and Green (Rheinwald and Green 1975) (Figure 1). Cells were maintained at 37 °C in a CO_2 (5%) incubator. The keratinocyte culture medium was a

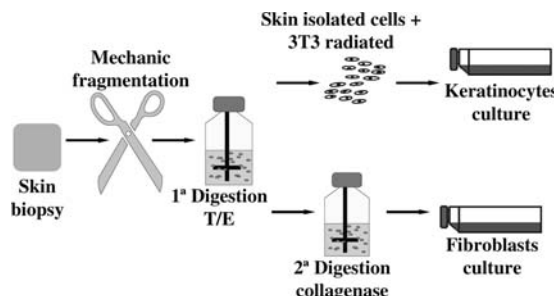

Figure 1. Obtaining cells for culture from a single small autologous biopsy.

mixture of DMEM/HAM-F-12 supplemented with 10% FCS, EGF, insulin, cholera toxin, hydrocortisone, triiode-thyronine and adenine as previously described (Cooper et al. 1991). The medium was changed every 48–72 h.

Fibroblast culture

Cells obtained after collagenase digestion were seeded (100,000 cells/cm^2) in the absence of lethally irradiated 3T3, using DMEM supplemented with 10% FCS as the culture medium (primary fibroblast culture) (Figure 1). The medium was changed every 72 h. Fibroblasts were subcultured until enough were obtained to prepare the plasma-based dermal equivalent. Fibroblasts were then detached from the flask by T/E treatment, counted and used as dermal cells in the dermal equivalent.

Preparation of plasma-based dermal equivalent

Fresh frozen plasma was obtained from voluntary donors from the local blood bank (CCST) using sodium citrate as anticoagulant agent, according to the standards of the American Association of Blood Banks (AABB) (Walker 1990). A plasma scaffold filled with autologous fibroblasts was used as the dermal component of the bioengineered skin. The plasma-based dermal equivalent was prepared as follows (Figure 2).

$6–7 \times 10^4$ cultured fibroblasts were resuspended in 10 ml of plasma containing 10 mg of tranexamic acid (antifibrinolytic agent Amchafibrin, Fides-Ecopharma), the final volume was adjusted to 23 ml by adding saline (NaCl 0.9%) and finally, 2 ml of $CaCl_2$ (1% in saline) were added to start the coagulation of the plasma. The mixture was placed in a tissue culture flask (75 cm^2) and allowed to solidify at 37 °C in a CO_2 incubator for 30 min. Once this dermis was solidified, it was covered with culture medium and 24 h later, cultured keratinocytes obtained after T/E digestion were seeded over its surface. Keratinocytes were seeded at different densities depending on the patient (between 5,000 and 12,000 cells per cm^2). When it was not necessary to seed all the cultured keratinocytes, the remaining cells were frozen using DMEM, 10% FCS and 10% glycerol as cryoprotector. Cells were frozen in cryovials at −1 °C/min, until −70 °C, the cryovials were maintained in a −70 °C freezer overnight and then transferred into a liquid nitrogen container for long-term storage. Cultures were observed by inverted microscope and when keratinocytes reached confluence (10–12 days), the culture flasks were opened, the culture medium taken out, the plasma-based bioengineered skin was fixed to a non-petroleum gauze with inorganic polymer glue (Histoacryl®, Braum) as previously described (Meana et al. 1997) and manually detached from the culture flask. The sheets were rolled up and introduced in a 50 ml tube containing DMEM medium and sent to the hospitals by express courier.

Patients

In the last 18 months, 20 biopsies coming from different hospitals across the country have been processed: 13 burned patients, 5 giant nevus, 1 graft vs. host disease (GVHD) and 1 neurofibromatosis.

In total 97,525 cm^2 of engineered skin were cultured. They were distributed by pathologies as

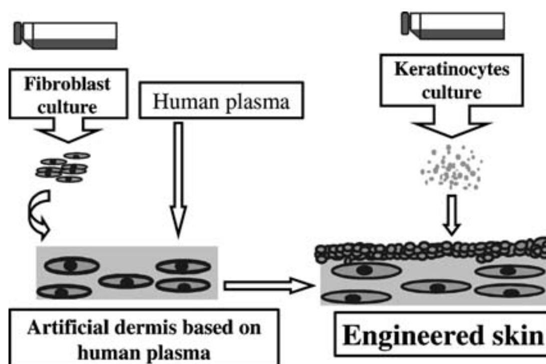

Figure 2. Preparation of plasma-based artificial skin.

follows: burned patients 83,575 cm^2, giant nevus 9,900 cm^2, GVHD 2,550 cm^2 and neurofibromatosis 750 cm^2.

In 7 cases, specimens of the autologous cultured skin were sent in the same transport conditions to the CIEMAT laboratory (located 500 km away from our bank) and grafted to athymic mice to prove the viability of the composite graft. Histological studies were performed pre and post-grafting (4 weeks).

Results

Biopsy collection

All biopsies were received and processed in less than 24 h after the harvesting. In all cases, enough cells were obtained to establish keratinocytes and fibroblast cultures (Table 1).

Engineered skin development

The culture period of this artificial skin model was 24–26 days. The biopsy size and the final cultured surface are shown in Table 1. Less than 1% of sheets sent to the different hospitals were broken during transportation. The experimental grafting on mice showed that skin took in all 7 cases, regardless of the result obtaining in the patient.

Patients

The engineered skin took in all patients, but the taking average was very different, depending on previous pathology. The epithelization obtained was permanent in all cases (Figure 3). During the follow up period (between 1 and 6 months: 6 patients, 7 and 12 months: 8 patients and 13 and 18 months: 6 patients) epithelial loss, blistering injuries or skin retractions were not observed. The aesthetic and functional results were acceptable.

Burned patients

The taking percentage in burned patients ranged between 10 and 90% (Table 1, taking percentage): 2 patients achieved a taking percentage ranged between 10 and 30%, 3 patients between 31 and

Table 1. Patients treated with this prototype of artificial skin for the last 18 months.

Patient	Age	Biopsy size (cm^2)	Cells obtained ($\times 10^6$)		Cultured surface grafted (cm^2)	Amplification	Take (%)
			Collagenase	T/E			
B1	33	6.5	10	8	4300	660×	30
B2	47	7	25	6	6525	930×	10
B3	8	3	16	5	1500	500×	90
B4	38	4.5	12	4	1875	400×	35
B5	62	10	25	12	4500	450×	35
B6	12	10	28	10	14,250	1400×	80
B7	13	9	14	3	9225	1000×	65
B8	8	3.5	8	5.5	3000	850×	80
B9	33	5	33	5	3000	600×	40
B10	20	6	12	2.5	9525	1500×	70
B11	50	7	17	4.5	6000	850×	60
B12	27	4.5	30	6	17,025	3700×	55
B13	18	8	36	6	2850	350×	90
N1	8	2	4	2	1875	900×	90
N2	10	3	4	2	1800	600×	70
N3	6	3	28	4	1650	550×	80
N4	9	3	13	2	1575	525×	85
N5	12	3	6	0.8	3000	1000×	85
GVHD	16	4	8	7	2550	630×	90
NF1	27	3.5	4	0.7	1500	420×	75

(B) Burned patients, (N) giant nevus, (GVHD) graft vs. host disease, (NF) neurofibromatosis. (Amplification = cultured surface grafted/biopsy size).

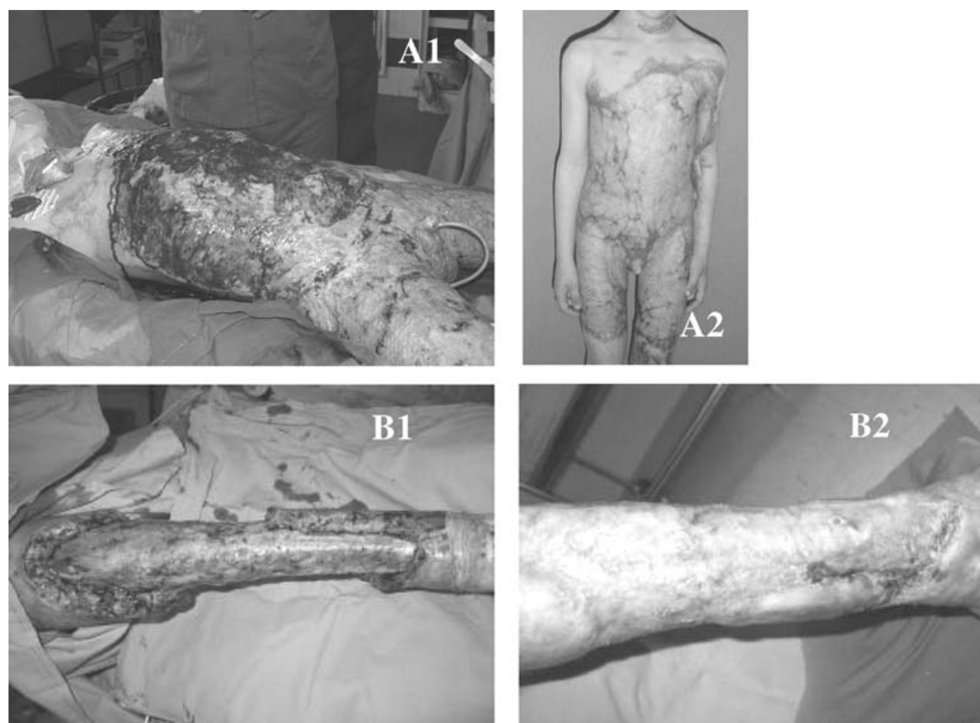

Figure 3. Patients treated with this model of artificial skin: (a) Burned patient, (b) GVHD (Graft vs. host disease). (1) Before treatment, and (2) after treatment.

50%, 4 patients 51 and 70% and 4 patients between 71 and 90%.

Non-critical patients

In the cases of giant nevus, the taking percentage was 70–90%. In the case of GVHD, a taking percentage of 90% was achieved. In the case of neurofibromatosis, a taking of 75% was achieved.

Discussion

One of the limitations in the treatment of severely burned patients is the lack of enough autologous epithelium to cover the wounds. This problem may be solved by tissue engineering techniques, which will be the base of future reparative medicine. In 1975, when Rheinwald and Green published their method for culture keratinocytes (Rheinwald and Green 1975), this problem seemed to be solved. Clinical experience with cultured epithelia autografts shows the limitations of this cultured tissue: sheets are fragile, the surface of the sheets decreases during the process and transplant, they have little resistance to slight traumatisms (Sheridam and Tompkins 1995). Moreover, the lack of an adequate dermal layer produces an abnormal dermo–epidermal junction (Woodley et al. 1988; Desai et al. 1991). The final result is the loss of the epithelization in treated areas and the formation of blistering lesions. The development of a dermal component for keratinocyte culture may solve the problems of Green's culture method, since it provides a keratinocyte support, which facilitates the grafting procedure, and also provides a dermal layer which facilitates the definitive taking of cultured epithelium (Boyce et al. 1993). However, the complete engineered skin models have problems to meet the great demand of cultured skin by severely burned patients.

In our centre, we have cultured keratinocytes over an artificial dermis based on human plasma and fibroblasts to avoid the fragility of the sheets, the lack of dermal component and to improve the results obtained by cultured epithelia autografts. The final product is a prototype, which contains both layers of the skin, dermis and epidermis. That is the reason why it can be called *Complete artificial skin*.

In this new system, both keratinocytes and fibroblast come from a minimal skin biopsy, this is possible due to the optimization of cell extraction from the biopsy. With the double enzymatic digestion, we obtain more cells (between 13 and 87%) than with the single T/E digestion. In this way, we can obtain enough cells to start both primary keratinocytes and fibroblasts culture. Cells coming from T/E digestion have been cultured following Green's method, giving as a result a keratinocyte culture; cells coming from collagenase digestion have been cultured in the absence of 3T3 cells, resulting in fibroblast culture.

In our model, human plasma has been used as a dermal scaffold to synthesize the artificial dermis. Plasma is a cheap product from blood banks, which is tested to avoid blood virus transmission. Moreover, human plasma can be stored frozen and quarantined to avoid the virus transmission risk during the windows period. Human plasma may also be used after methylene blue viral inactivation without changing its properties as a dermal matrix. All this, along with the great clinical experience using human plasma, makes it an extremely safe product. Human plasma is an excellent dermal scaffold since it allows both keratinocytes and fibroblasts to grow rapidly. This fact makes it possible to start the secondary culture with an initially low cell concentration (keratinocytes and fibroblasts) and 10–12 days later to obtain a confluent layer of keratinocytes, over the plasma surface filled with an elevated concentration of fibroblasts in the plasma-based matrix. These properties allow an expansion rate enough to use this culture system to treat large skin lesions (amplification, Table 1).

This skin prototype is made of autologous keratinocytes and fibroblasts. Autologous keratinocytes are needed for the definitive epithelial coverture, but the presence of autologous fibroblasts can also repair the injured dermis (Svensjo et al. 2002; Wisser and Steffes 2003; Llames et al. 2004) since fibroblasts stay definitively in the wound bed, as it has been proved in the

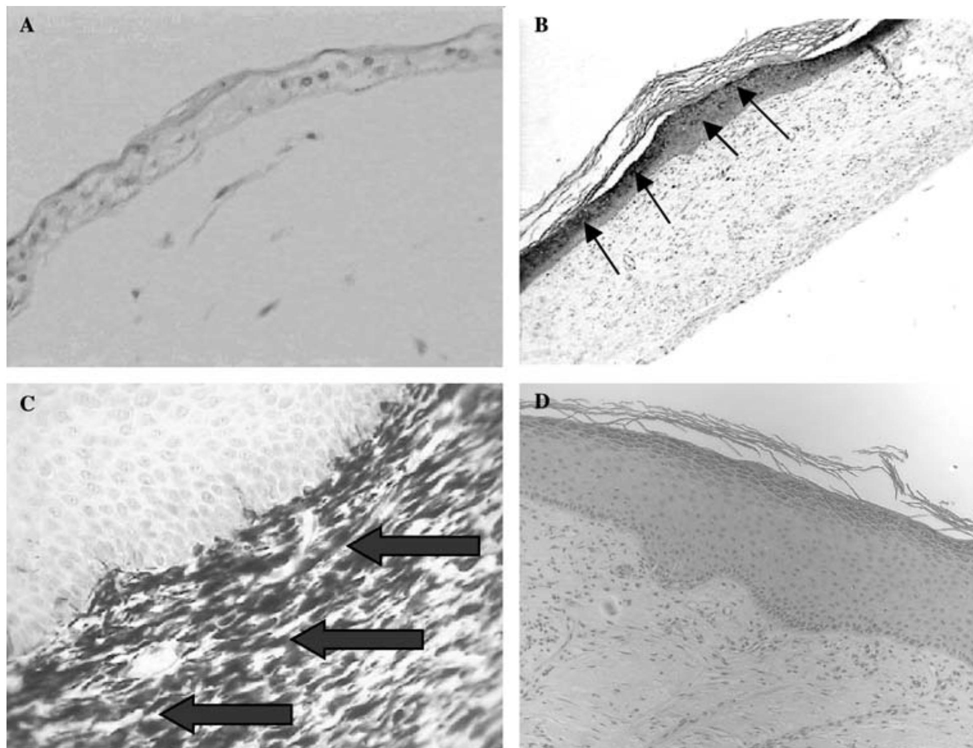

Figure 4. Histological results of artificial skin based on human plasma. (a) H & E of this prototype before grafting, 200×. (b) Human involucrin inmunostaining (Clone SY5, Sigma), 4 weeks after grafting onto athymic mice, 100× . (c) Human vimentin inmunostaining (V9, BioGenex, San Ramon, CA), 4 weeks after grafting onto athymic mice, 400×. (d) H & E, 8 weeks post-grafting onto burned patient, 100×.

experimental grafting of this skin equivalent to athymic mice (Figure 4).

The clinical grafting of this prototype shows the variability in the taking average between burned and non-critical patients. The taking percentage in burned patients ranged between 10 and 90%. This variability was mainly due to frequent complications in burned patients like: hemodynamic descompensations, sepsis and infected wound beds. In the case of non-critical patients, the taking percentage increased with respect to the burned patients. In this case the surgical procedure was elective and programmed, biopsies were taken well in advance and the grafting procedure was carried out in the same surgical procedure in which the lesion was excised, so wound beds did not have infectious or hemodynamic problems. These clinical conditions are similar to experimental grafting in athymic mice, where the taking average was close to 100%.

This work not only shows the clinical results of a new model of complete artificial skin but also demonstrates that it is possible to produce this prototype in high quantity, to transport it in good viability conditions and to distribute it to different hospitals far from our region.

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