Simultaneous Isolation of Keratinocytes and Fibroblasts from a Human Cutaneous Biopsy for the Production of Autologous Reconstructed Skin

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The key step in the reconstruction of skin by the self-assembly approach is to use fibroblasts capable of secreting a mature extracellular matrix and keratinocytes that can associate with one another to form a stratified, differentiated epidermis. To determine the most efficient way to extract both cell types from a single cutaneous biopsy, five different enzymatic combinations were tested. The yield, viability and morphology of the recuperated cells were compared. The length and temperature of the enzymatic treatments influenced the number and the type of cells recuperated. The cells were then cultured in appropriate media in monolayers and reconstructed skin were produced using the self-assembly approach. No difference was observed between the different strains of cells.

La réussite de la technique d'auto-assemblage pour la reconstruction de la peau dépend de la capacité des fibroblastes à sécréter une matrice extracellulaire complexe ainsi que celle des kératinocytes à s'associer pour former un épiderme stratifié et différencié. Pour déterminer la manière la plus efficace d'extraire ces deux types cellulaires d'une seule biopsie cutanée, cinq combinaisons d'enzymes ont été comparées. La durée et la température des traitements enzymatiques ont influencé le nombre de cellule récupérées. Les cellules ont d'abord été cultivées en monouche, puis peaux reconstruites par la méthode d'auto-assemblage ont été produites. Aucune différence n'a été observée entre les différentes lignées.

Keywords: tissue engineering, reconstructed skin, keratinocytes, fibroblasts, culture.

The physico-chemical properties of a dermal-epidermal reconstructed skin provide numerous advantages for their use as permanent skin replacement over full thickness skin injuries. The reconstructed skin elaborated in our laboratory by the new tissue engineering technique, the self-assembly approach, possesses a network of elastic fibers and collagen fibrils that confers suppleness and strength to the reconstructed tissue (Larouche et al., 2000). This tissue engineered skin has tremendous therapeutic values for autologous grafting of large burn wounds, giant nevi, and the healing of cutaneous ulcers.

Our dermal-epidermal living human substitute is composed of skin fibroblasts that secrete their own extracellular matrix and skin keratinocytes that proliferate and differentiate into a cornified, stratified epithelium (Michel et al., 1999). No synthetic or exogenous extracellular matrix, such as collagen or fibrin, has been added to the organotypic culture. The only extracellular matrix and basement membrane material found in our reconstructed skin has been secreted, and assembled by the autologous cells in culture and is thus fully immunocompatible with the donor. The cells that constitute this reconstructed skin retain the ability to proliferate, differentiate, and heal (Laplante et al., 2000), which is of crucial importance in the long-term fonctionnality of the graft. The ability to secrete soluble factors enabling wound healing is a key function that is important for the use of our reconstructed skin as a wound-healing device.

The keratinocytes are usually isolated from skin biopsy using an enzymatic digestion with thermolysin or dispase and/or trypsin (Germain et al., 1993; Martinet et al., 1988; Rheinwald et al., 1975; Liu et al., 1978). The major obstacle in the isolation of epithelial cells is the contamination with fibroblasts which can overgrow the keratinocytes. The fibroblasts can be cultured from explants, but this method is time-consuming and not always successful. The mesenchymal cells can also be recuperated with a collagenase (enzymatic) treatment of the peeled dermis, and thus generating a more representative cell population. The simultaneous isolation of keratinocytes, fibroblasts, and endothelial cells using trypsin to digest the basal membrane, and explants to isolate fibroblasts has been documented (Normand et al., 1995). Their ability to reconstruct a tissue-engineered skin has not been investigated.

In order to use the self-assembly technique in autologous grafting, we had to develop a rapid and reproducible method for the isolation of the two cell populations (fibroblasts and keratinocytes) from a small skin biopsy. The isolated skin cells would also

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have to be able to proliferate, and, when cultured in the appropriate conditions, differentiate into a mature reconstructed skin. We already developped a successful method for the isolation of pure cultures of keratinocytes (Germain et al., 1993) by digesting the skin's basal membrane with thermolysin. We wanted to optimize a sequential enzymatic treatment by first isolating epidermal cells with thermolysin and then the fibroblasts by digesting the dense mesenchymal extracellular matrix with a collagenase. We tested five enzymatic combinations to isolate the keratinocytes and fibroblasts, and compared the yield and morphology of the isolated cells. Following cell amplification in monolayer cultures, reconstructed skin were produced by our self-assembly technique and their properties were studied.

Material and Methods

Cell Isolation

A normal skin biopsy sample from a reductive breast surgery was thoroughly washed in a phosphate buffered saline (0.14 M NaCl; 2.68 mM KCl; 8.1 mM Na_2HPO_4; 1.47 mM KH_2PO_4; 100 IU/mL penicillin; 25 µg/mL gentamycin; 0.5 µg/mL fungizone; pH 7.4) and cut into 1 cm^2 pieces. The tissue was transferred into a preheated (37°C) or cold (4°C) sterile thermolysin solution (500 µg per mL, Sigma, Oakville, Canada) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (6.7 mM KCl; 142 mM NaCl; 10 mM HEPES; 1 mM $CaCl_2$; 0.45 mM NaOH, pH 7.4) (Germain et al., 1993). The samples were incubated between 2.5 and 16 h under constant agitation (Table 1). After this first incubation, the epidermis was gently peeled from the dermis with forceps. The epidermis was incubated in a preheated trypsin (0,05%)-EDTA (0,01%) solution (Sigma) in phosphate buffer saline (0.14 M NaCl; 2.68 mM KCl; 8.1 mM Na_2HPO_4; 1.47 mM KH_2PO_4; 2.78 mM glucose) under constant agitation for 15 min. The epidermal cell suspension, mostly keratinocytes, was centrifuged (400 x g), counted with a hematocytometer, and plated. The dermis was incubated in a preheated (37°C) sterile collagenase H (0,125 U/ mL, Boerhinger Mannheim, GmbH) solution diluted in the fibroblast's culture medium (Dulbecco-Vogt modification of Eagles medium (Life Technologies, Grand Island, USA), supplemented with 10% fetal calf serum (Hyclone, Logan, USA), 100 IU/mL penicillin and 25 µg/mL gentamycin (Sigma) between 2.5 and 16 h under constant agitation (Table 1). The dermal cell suspension (fibroblasts) was centrifuged (400 x g), counted with a hematocytometer, and plated. This experiment was conducted once.

Fibroblast and Keratinocyte Cultures

Fibroblasts were plated at 40000 cells/cm^2 and cultured in fibroblasts' medium (Dulbecco-Vogt modification of Eagles medium, supplemented with 10% fetal calf serum, 100 IU/mL penicillin and 25 µg/mL gentamycin). Epidermal cells were plated at 13000 cells/cm^2 on a 3T3 irradiated feeder layer (20000 cells per cm^2) and cultured in keratinocytes' medium (Dulbecco-Vogt modification of Eagles medium (DME) with Ham's F12 in a 3:1 proportion (Life Technologies), supplemented with 10 ng/mL epidermal growth factor (EGF, Austral Biologicals, San Ramon, USA), 24.3 µg/mL adenin, 5 µg/mL insulin, 5 µg/mL transferrin, 2×10^{-9} M 3,3', 5', triiodo-L-thyronine (Sigma), 0.4 µg/mL hydrocortisone (Calbiochem, La Jolla, USA), 0.2 mg/mL isoproterenol (Sabex, Boucherville, Canada), 5% fetal calf serum (Hyclone), 100 IU/mL penicillin G and 25 µg/mL gentamicin). Media were changed three times a week. Fibroblasts and keratinocytes were trypsinized once before they were used in the production of the reconstructed skin.

Reconstructed Skin Production

Fibroblasts were cultured 35 d in the presence of 50 µg/mL of ascorbic acid (Sigma) to form sheets. Three fibroblast sheets were piled up to form the reconstructed dermis. The keratinocytes were seeded on top and allowed to grow to confluence. The dermal-epidermal substitute was brought to the air–liquid interface for 14 d to allow the maturation of the epidermis.

Histologic Analysis

The reconstructed tissues were fixed in a Bouin solution (ACP, Montréal, Canada). Skin samples were fixed in Histochoice (Amresco, U.S.A.) after digestion. Cross-sections (5 µm) of the paraffin-embedded tissues were stained with Masson's trichrome.

Electron Microscopy

The reconstructed tissues were fixed 24 h in 0.1 M cacodylate solution containing 2.5% glutaraldehyde (Mecalab, Montréal, Canada). The samples were post-fixed in a 1% OsO_4 solution, dehydrated in ethanol and embedded into epoxy. Thin cross-sections (60-80 nm) were stained with uranyl acetate and lead citrate, and observed on a JEOL 1200 EX electron microscope.

Results

A skin sample from a reductive breast surgery was cut into five areas of 1 cm^2 each and submitted to a combination of enzymatic digestions (refer to Table 1). Additional skin was cut into pieces and digested in thermolysin under the same conditions. Theses pieces were then fixed for histologic analyses.

Histologic cross-sections of the skin samples treated with thermolysin show a good separation between the dermis and the epidermis for the conditions A, B, D or E (refer to Table 1). Thermolysin has digested the majority of the basal membrane between fibroblasts and keratinocytes in either 2.5 h at 37°C or 10 h at 4°C. On the other hand, the epidermis of the skin sample digested in condition C was difficult to peel, indicating an incomplete digestion of the keratinocyte basal membrane. Histologic cross-section of the skin digested with condition C confirms this incomplete digestion (arrow on Figure 1c). It seems as though less than 8 h in the cold (4°C) thermolysin solution is insufficient to digest the entire basal membrane proteins and liberate the epidermal cells from the dermis. Therefore, the skin must be incubated 2.5 h at 37°C or 10 h at 4°C in the thermolysin solution for the complete digestion of its dermo-epidermal junction.

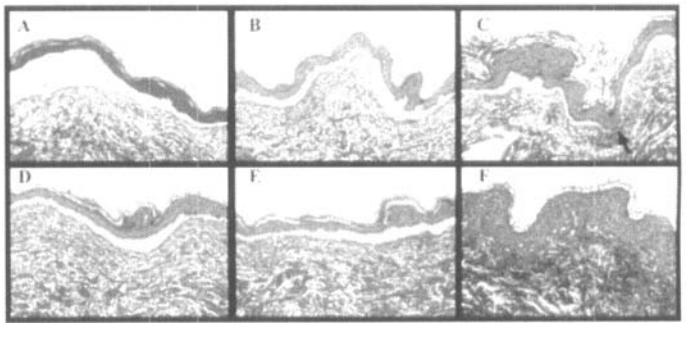

Figure 1. Histologic cross-sections (Masson's trichrome staining) of skin samples after incubation in thermolysin for various times as described in Table 1. (a) Condition A; (b) Condition B; (c) Condition C; (d) Condition D; (e) Condition E (f) undigested normal human skin. The arrow in C points to an undigested portion of the basal membrane.

Table 1. Enzymatic treatments tested for the digestion of the skin biopsy and yields in viable fibroblasts and keratinocytes.

Enzymatic digestions	Keratinocytes per cm^2	Fibroblasts per cm^2
A Thermolysin 2.5 h at 37°C		
Trypsin 0.25 h at 37°C	3 700 000	
Collagenase H 2.5 h at 37°C		4 600 000
B Thermolysin 2.5 h at 37°C	N. D.*	
Collagenase H 16 h at 37°C		2 000 000
C Thermolysin 8 h at 4°C		
Trypsin 0.25 h at 37°C	3 200 000	
Collagenase H 16 h at 37°C		2 000 000
D Thermolysin 10 h at 4°C		
Trypsin 0.25 h at 37°C	6 200 000	
Collagenase H 10 h at 37°C		3 100 000
E Thermolysin 16 h at 4°C		
Trypsin 0.25 h at 37°C	5 400 000	
Collagenase H 2.5 h at 37°C		3 000 000

*N.D. = Not determined

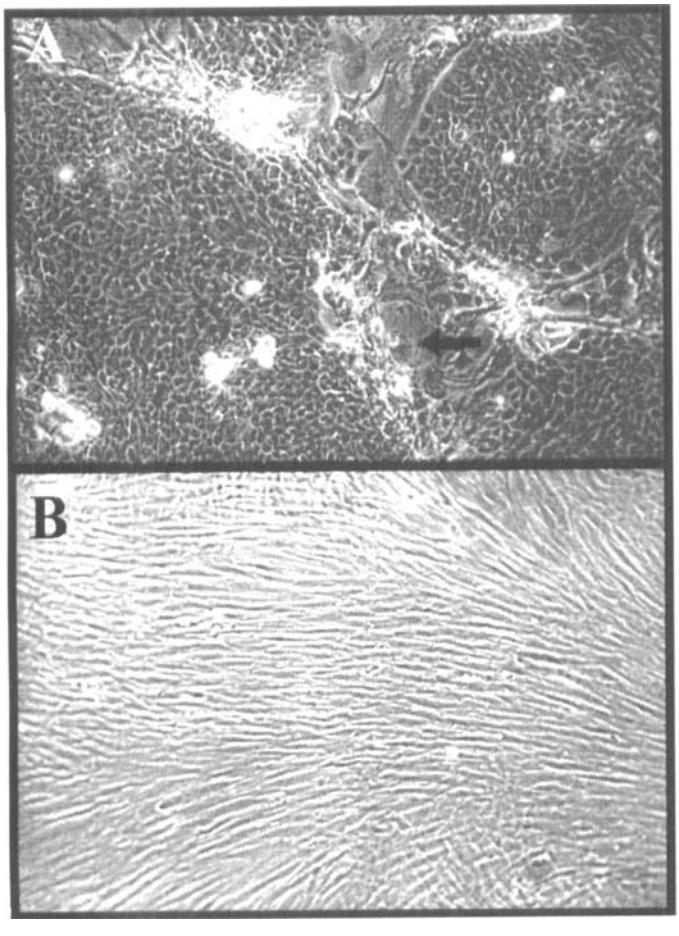

Figure 2. Phase contrast morphology of (a) keratinocytes' and (b) fibroblasts' primary cultures after, respectively, 10 and 12 d in culture. The arrow in (a) points to the remnants of irradiated fibroblasts feeder layer.

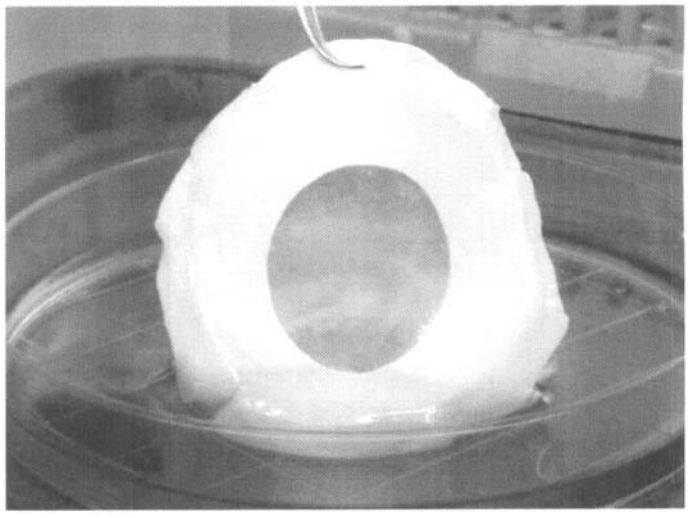

Figure 3. Macroscopic appearance of the reconstructed skin cultured at the air–liquid interface for 14 d.

After the treatment of the skin with the thermolysin solution, the epidermis was gently peeled and the keratinocytes were digested with trypsin. The recuperated cells were counted with an hemacytometer and cultured in the appropriate medium. The greatest keratinocyte yield was obtained when the thermolysin digestion was performed at 4°C at least for 10 h (Table 1). Longer incubation in thermolysin did not significantly increase the number of recuperated epithelial cells. Short incubation (2.5 h at 37°C) of the tissues in the preheated thermolysin solution reduced slightly the yield in keratinocytes.

The dermis obtained from the thermolysin treatment was incubated in a collagenase H solution under constant agitation. The cells released from the dermis were counted and plated. The greatest number of fibroblasts were recuperated in condition A, after a 2.5 h incubation in the collagenase H solution. Prolonged incubation (more than 10 h) in the collagenase H solution greatly reduced the number of fibroblasts.

The keratinocytes and fibroblasts cultures of conditions A, D and E were free of any contaminants and adopted the expected morphology (Figure 2). Keratinocytes are small polygonal cells able to form colonies when seeded on a feeder layer (Figure 2a). Keratinocytes are trypsinized when they reach about 80% confluence between 7 and 10 d in culture. In primary culture (P0), the density of keratinocytes is the highest in condition E, intermediate in condition A, and the lowest in condition C and D (Table 2). After one passage (P1) the number of cells obtained per square centimeter was similar for conditions A, D, and E, and slightly lower for condition C, even though the cells were seeded at the same concentration (Table 2).

Fibroblasts are elongated cells (Figure 2b) that reached confluence between 7 and 12 d. The fibroblasts isolated from condition B and C did not grow properly; after 20 d in culture, they did not reach confluence. After 12 d in primary culture, the fibroblasts from conditions A, D, and E were trypsinized at confluence. Numerous cells were recuperated from the monolayer culture of condition A, while the cultures from condition D or E contained far less cells (Table 2). After one passage, the fibroblastic cultures were more homogeneous (Table 2).

Table 2. Number of keratinocytes and fibroblasts obtained during culture.

Type of cells	Conditions used for their isolation[1]	Number of cells per cm^2 in primary culture	Number of cells per cm^2 after one passage
Keratinocytes[2]	A	210000	180000
Keratinocytes	C	200000	150000
Keratinocytes	D	200000	190000
Keratinocytes	E	240000	180000
Fibroblasts[3]	A	70000	36000
Fibroblasts	B	N.A[4]	N.A.
Fibroblasts	C	N.A.[4]	N.A.
Fibroblasts	D	35000	27000
Fibroblasts	E	21000	37000

[1] Refer to Table 1 for a descripion of the conditions.
[2] Keratinocytes were trypsinized when 80% confluence was reached in condition A after 10 d for primary culture and 7 d after one passage. The epidermal cells were seeded at 13000 cells per cm^2 on a feeder layer.
[3] Fibroblasts were trypsinized when cells of the condition A reached confluence after 12 d for primary cultures and 7 d after one passage. For each condition, fibroblasts were seeded at 40000 cells per cm^2 in primary culture, and at 7000 cells per cm^2 for the first passage.
[4] The fibroblasts generated in condition B and C did not grow properly (5% confluence after 20 d in culture); they were not trypsinized.

The epithelial and fibroblastic monolayer cultures from conditions A, D and E were trypsinized once and used for the production of reconstructed skins by the self-assembly technique. All reconstructed skins were comparable, independantly of the conditions used to isolate both cell types (macroscopic view of the reconstructed skin in Figure 3). The dermal moiety was composed of fibroblasts embedded into an extracellular matrix (blue stain in Figure 4a). The presence of a well organized extracellular matrix confers the reconstructed skin resistance, elastic properties and suppleness. The keratinocytes were adequately organized into a differentiated, cornified epithelium (Figure 4a). The cornification of the epithelium confers a mechanical barrier against environmental insults and water loss on the reconstructed skin. The presence of an organized basal membrane at the dermo-epidermal junction (Figure 4b) enables the keratinocytes to firmly stick to the underlying stroma. The collagen fibrils formed a dense network as visualized by electron microscopy (Figure 4c).

Discussion

The main goal of this study was to find the right combination of enzymatic digestions to isolate functional fibroblasts and keratinocytes from a single skin biopsy and to test their ability to self-assemble into a mature human reconstructed skin. The cell cultures obtained by the various digestions were not cross contaminated by the other cell type (i.e. no keratinocytes in fibroblasts cultures, and vice versa). The cells isolated in conditions A, D and E tested retained their ability to proliferate and differentiate into a reconstructed skin.

The main difference between the conditions tested was the number of cells recuperated. For the keratinocyte isolation, incubation in a thermolysin solution for 10 h at 4°C (condition D) yielded the greatest number of epithelial cells, even though a clear separation between the dermis and the epidermis was observed when the skin was digested 2.5 h in a preheated thermolysin solution (condition A) or 16 h in a cold enzymatic solution (condition E).

The incomplete digestion observed in condition C has lead to a decrease in the density of keratinocytes during culture. Skin stem cells rest in the basal layer of the epidermis and cells from

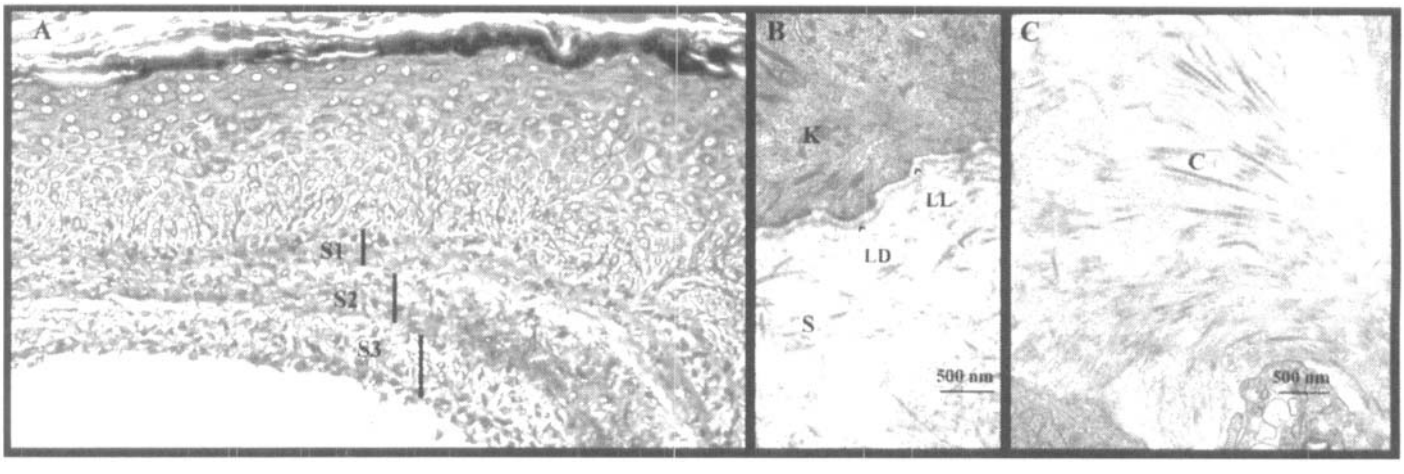

Figure 4. (a) Histologic staining (Masson's trichrome) showing the presence of collagen fibrils (in blue) in the dermis of the reconstructed skin. The superposed fibroblast's sheets (S1, S2 and S3) can be visualized. (b) Ultrastructural picture of the reconstructed skin showing the basal membrane between keratinocytes and (c) the underlying stroma. K, keratinocytes; LL, Lamina lucida; LD, lamina densa; S, stroma; C, collagen fibrils.

this layer are partially lost with an incomplete digestion. We conclude that the condition C is not suitable for cell isolation since skin stem cells may not be fully recuperated. The stem cells are necessary for long-term skin regeneration and its healing after wounding of the epidermis. Indeed, stem cells are important players in a tissue-engineered skin since they serve to continually provide new epithelial cells for the formation of a stratified, cornified epithelium.

On the other hand, long incubation (more than 8 h) in thermolysin reduced the number of the recuperated fibroblasts. These results may be explained by the absence of serum (and growth factors) in the thermolysin solution, or the potential cytotoxicity of thermolysin to mesenchymal cells leading to an increase mortality of the fibroblasts.

For the fibroblast isolation, lengthy treatments (more than 10 h) in the collagenase solution significantly reduced the number of recuperated viable cells. Fibroblasts obtained from a 16 h collagenase H digestion (condition B and C) were not able to proliferate in culture. Incubation at 37°C enables the optimal activity of the enzyme but can also cause stress and alter the cell viability. Dying cells may release the content of their cytoplasm and some proteins (such as proteases) in the collagenase H solution, and thereby may harm viable fibroblasts being digested from the extracellular matrix.

The rate-limiting step in the production of our skin equivalent is the production of the fibroblasts' sheets that requires plating the greatest number of cells as soon as possible. Since all the conditions used here generated cells able to recreate into an histologically adequate reconstructed skin, secrete and reorganize a complex extracellular matrix and a specialized basal membrane, we think that condition A is the most valuable choice. This sequential combination of enzymatic incubation (thermolysin 2.5 h at 37°C; trypsin 15 min at 37°C; collagenase H 2.5 h at 37°C) allowed us to recuperate the two cell populations the day of the biopsy with a maximal number of fibroblasts.

Our results demonstrate the importance of the incubation time and temperature in the recuperation of skin cells. They also show the feasibility of isolating two cell types from a single skin biopsy, and culturing those cells to recreate an autologous human reconstructed skin. Our reconstructed skin is presently used for the treatment of difficult-to-heal venous leg ulcers to enhance the reepithelialization of the wounds. It could also be used in the treatment of burn victim, in conjunction with other treatment such as epidermal sheets, to enhance would healing and reduce scarring.

Conclusion

In conclusion, we have developed a technique to isolate both human keratinocytes and fibroblasts from a single biopsy. The following sequential enzymatic treatment was preferred: thermolysin 2.5 h at 37°C followed by trypsin 15 min at 37°C; and collagenase 2.5 h at 37°C. It allowed the isolation of both cell types rapidly within 8 h following the biopsy. Furthermore, fibroblasts were adequate for the production of reconstructed dermis by the self-assembly approach. After keratinocyte culture on the reconstructed dermis, a reconstructed skin histologically similar to normal human skin was successfully obtained.

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