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Reconstructed skin from co-cultured human keratinocytes and fibroblasts on a chitosane cross-linked collagen-GAG matrix

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Reconstruction of skin requires both the dermal and epidermal equivalent of the skin. A reconstructed skin has been developed, composed of two compartments: (1) a dermal equivalent (DE) comprising an acellular dermal substrate (DS) populated by foreskin fibroblasts (FF); (2) an epidermis regenerated from normal human keratinocytes (NHK) seeded on to the DE. The DS contains Types I and III collagen and glycosaminoglycans (GAGs) cross-linked by chitosane. FF seeded into the porous structure of the DS provide a DE suitable to support epidermal cells. NHK attach quickly, exhibit mitotic activity and form a continuous and stratified epidermis. After 2 weeks culture, histological sections of the RS show a basal layer with cuboidal cells attached to the DE and several suprabasal cell layers including the stratum corneum (SC). Transmission electron microscopy revealed the cell membrane densification (hemidesmosomes) at the dermal–epidermal junction; however, the lamina densa was found to be discontinuous at this stage. We noted the presence of lipid vesicles in spinous layer and keratohyalin granules in granular layer. Terminal epidermal differentiation was complete with the SC consisting of several layers of corneocytes filled with tonofilaments. Immunofluorescence studies revealed the presence of bullous pemphigoïde antigen and Type IV collagen at the dermal–epidermal junction as well as the presence of keratins 1/10 and filaggrin in the suprabasal layers characteristic for epidermal differentiation. Reconstructed skin based on our chitosane cross-linked collagen–GAG matrix is morphologically equivalent to normal human skin and should thus provide a useful tool for the treatment of patients with severe burns.

1. Introduction

Large thermal or chemical burns, traumatic degloving injuries, as well as pathological conditions, often require a large amount of skin grafts for wound closure. In extensive burns, autografts are insufficiently available and skin removal inflicts additional injury at the donor site.

Alternatives to split thickness skin have been studied by several groups in Europe, including partial (epidermis or dermis) or complete (epidermis and dermis) replacement. Several types of skin substitute have been developed, based on pure cultured normal human keratinocytes (NHK) [1–3], NHK or skin explants cultured on collagen gels contracted by human fibroblasts [4, 5], and NHK cultured on collagen glycosaminoglycans (GAG) populated with normal human fibroblasts (NHF) [6, 7].

The reconstruction of skin from co-cultured human keratinocytes and fibroblasts on a dermal substrate (DS) made of a chitosane cross-linked collagen–GAG matrix, is reported here. This reconstructed skin thus comprises two compartments, a dermal equivalent composed of the DS populated by fibroblasts, and an epidermis regenerated from NHK seeded on the DE.

2. Materials and methods

2.1. Preparation of dermal substrate (DS)

Preparation of artificial dermis has been described previously [8]. Briefly, bovine collagen Types I and III was dissolved in 0.05 M acetic acid. Chitosane (deacetylated chitin) and glycosaminoglycans (GAG: chondroïtin-4 sulphate and chondroïtin-6 sulphate), were dissolved and added to the collagen gel.

The final preparation was cast into sheets and lyophilized overnight. Dry membranes were then sterilized by gamma-irradiation (25 kGy) and stored in 70% ethanol until use.

2.2. Preparation of dermal equivalent (DE)

For cell culture, DS was first washed in phosphate-buffered saline (PBS), then equilibrated by three baths in a culture medium.

Subcultured foreskin fibroblasts (5×10^5 cm^{-2}) were seeded on the porous structure of DS in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS) and incubated at 37 °C in 5% CO_2 for 2 weeks.

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2.3. Keratinocytes culture on DE

Subconfluent NHK (second passage) were seeded on to the DE (4×10^5 cm^{-2}) in serum-free MCDB 153 medium supplemented with insulin (5 μg ml^{-1}), hydrocortisone (0.4 μg ml^{-1}), EGF (10 ng ml^{-1}). After 2–4 days incubation, the DE was elevated to the air–liquid interface using a stainless steel grid and was incubated for a further 2 weeks.

2.4. Characterization of reconstructed skin (RS)

After 14 days, the samples were fixed in 10% formalin and embedded in paraffin. Sections 5 μm thick were stained with haematoxylin–eosin–saffron. Slides were examined under Zeiss IM 35 microscope.

2.5. Electron microscopy

For scanning electron microscopy (SEM), dry samples of DS were sputter-coated with gold–palladium and observed under a Hitachi S800 microscope to determine the pore size.

For transmission electron microscopy (TEM), RS was washed rapidly with PBS, fixed in 2% glutaraldehyde in phosphate buffer (0.1 M), pH 7.4, for 2 h at room temperature, rinsed overnight at 4 °C in phosphate buffer and then post-fixed in 1% osmium tetroxide for 2 h, dehydrated in alcohol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate before examination with a Philips EM 300 microscope. SEM and TEM observations were realized in the CMEABG (Université Claude Bernard, Villeurbanne, France).

2.6. Indirect immunofluorescence studies

Unfixed samples were rinsed in PBS, embedded in Tissu-Teck OCT compound, frozen in liquid nitrogen and stored at − 80 °C until used.

Vertical frozen sections (5 μm) of tissue samples were processed for indirect immunofluorescence studies.

Bullous pemphigoïd sera were obtained from Dr J. H. Saurat (Geneva, Switzerland). A mouse monoclonal antibody against human collagen IV was used.

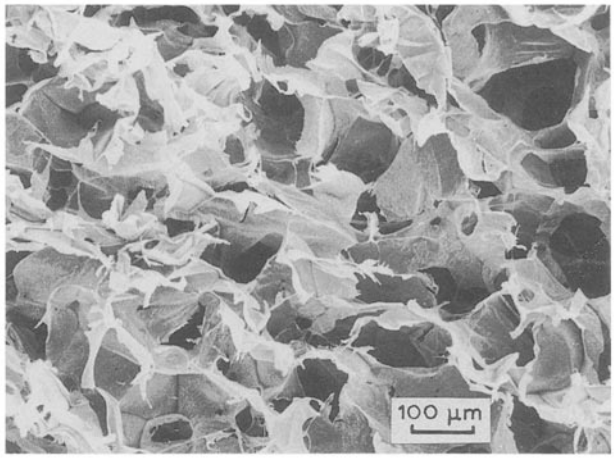

Figure 1 Scanning electron micrograph of a cross-section of lyophilized DS. Note the homogeneous porous structure.

Antibody K 8-60 against keratins 1/10, a polyclonal antibody against human filaggrin, and fluorescein conjugates were also used.

3. Results

3.1. Pore size control of DS

Scanning electron microscopy of a section of DS revealed a homogeneous porous structure with pores ranging from 50–120 μm (Fig. 1). Furthermore, a difference was observed in the ultrastructure between the side which was in contact with the air, and that which was in contact with the freeze-drying pan during lyophilization of DS. The air-exposed side was less porous with smaller pores than the "pan" side (Fig. 2a, b). Consequently, it was decided to seed the fibroblasts to the "pan" side and keratinocytes on to the "air" side.

3.2. Histology

The histological appearance of RS (Fig. 3) shows that fibroblasts invaded DS and subsequently elongated and spread by orientating their long axis parallel to neighbouring fibroblasts. They form a network with collagen–GAG providing a dermal equivalent beneath the keratinocytes. NHK adhere to the DE and form a

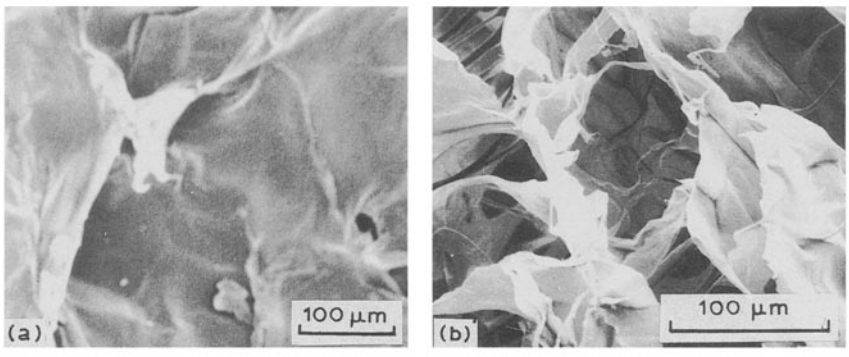

Figure 2 Scanning electron micrographs of lyophilized DS: (a) the air-exposed side and (b) the side which was in contact with the freeze-drying pan during lyophilization of the DS.

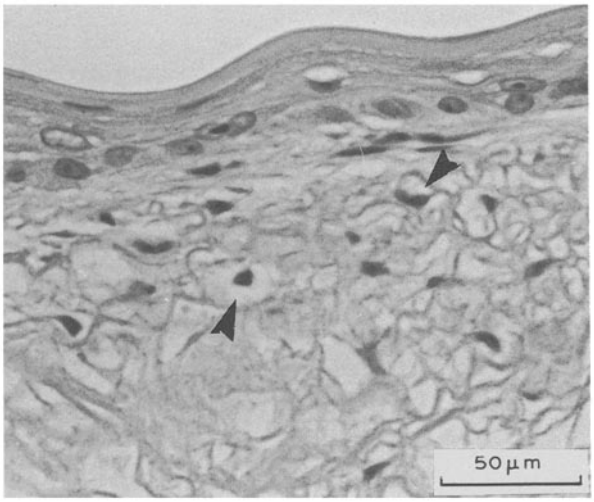

Figure 3 Photomicrograph of hematoxylin–eosin–saffron-stained reconstructed skin after 18 days culture. Arrows: fibroblasts that form a mesh-work with collagen–GAG matrix beneath the keratinocytes. NHK form a continuous and stratified epidermis with cuboidal basal cells attached to the matrix, flattened suprabasal cells and a stratum corneum. Note the presence and orientation of fibroblasts at the dermoepidermal junction.

basal layer with cuboïdal cells and multiple suprabasal layers including stratum corneum (SC).

3.3. Transmission electron microscopy

Transmission electron micrographs show that NHK form stratified epidermal layers with all characteristic cell layers: basal, spinous, granular as well as cornified layers. It was possible to see the cell membrane densification (hemidesmosomes) and the formation of lamina densa-like material at the dermal–epidermal junction (DEJ) (Fig. 4). The presence of well-organized desmosomes in all layers, lipid vesicles in a spinous layer (Fig. 5) and keratohyalin granules in granular layers (Fig. 6) was noted. The epidermal differentiation was fully terminal with the SC containing several layers of corneocytes filled with tonofilaments (Fig. 7).

3.4. Indirect immunofluorescence studies

The distribution of basement membrane antigens at the DEJ of RS is shown in Fig. 8. The bullous pemphigoïd antigen has both an intracellular localization

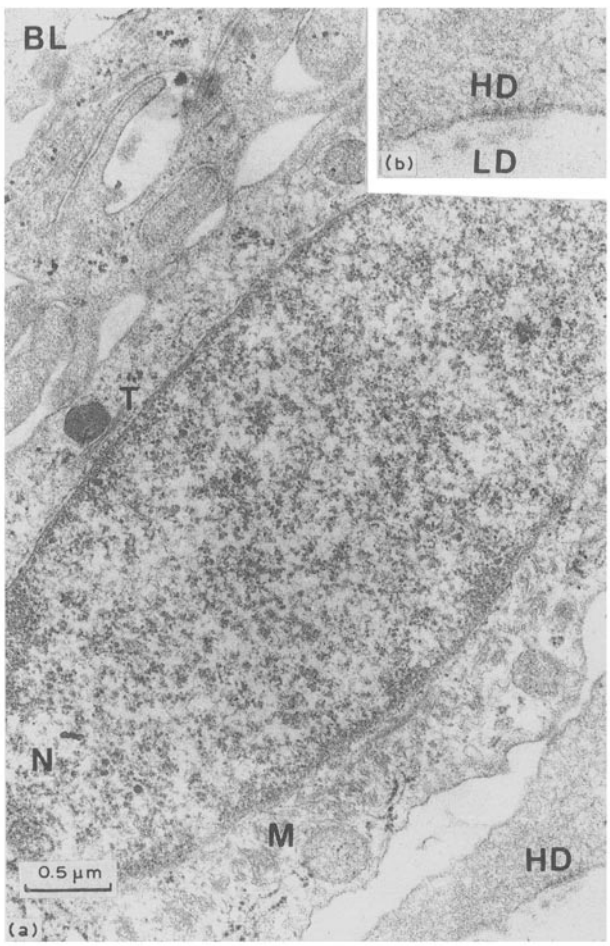

Figure 4 (a) Transmission electron micrograph of the reconstructed basal layer (BL). Note the oval-shaped nucleus (N), the tonofilaments (T), the mitochondria (M) in the basal cell, the hemidesmosome (HD) at the dermoepidermal junction as well as the discontinuous lamina densa (LD). (b) Enlargement of HD and LD: × 54 000.

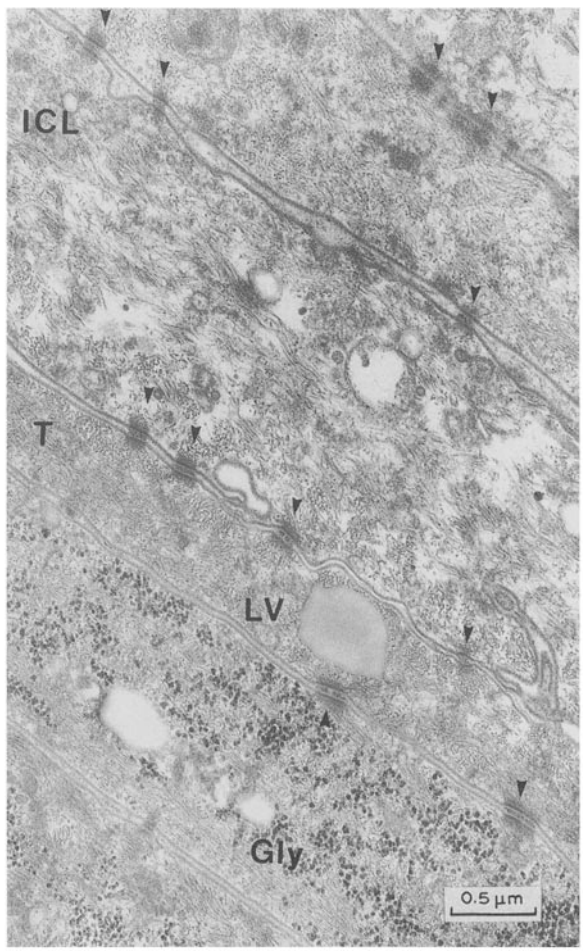

Figure 5 Transmission electron micrograph of the intermediate cell layers (ICL) in the reconstructed epidermis with frequent well-organized desmosomes (arrows) that connect the epidermal cells. Note the numerous tonofilaments (T), the lipid vesicle (LV) and the glycogen (Gly) in the ICL.

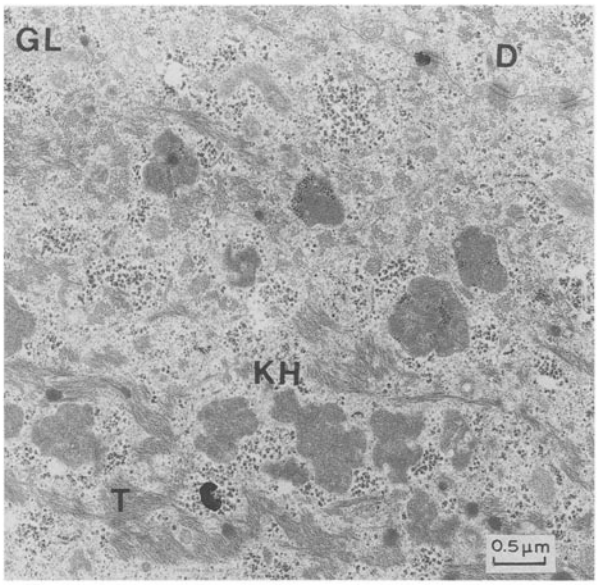

Figure 6 Transmission electron micrograph of the reconstructed granular layer (GL) with polymorphic and irregularly shaped keratohyalin granules (KH). Note the clusters of ribosomes in the immediate vicinity of KH. Tonofilaments (T) are frequently observed in the vicinity of the KH and on the right top well-organized desmosomes (D).

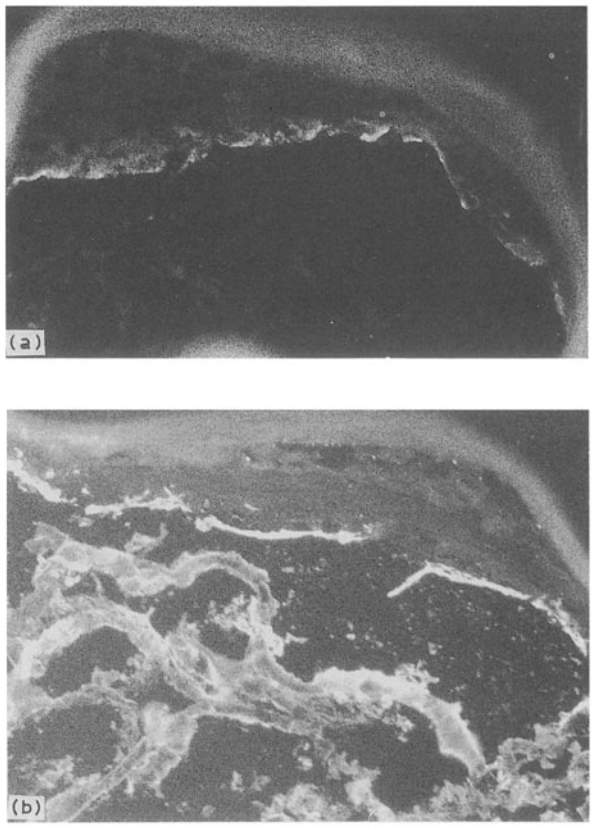

Figure 8 Immunofluorescence studies of reconstructed skin: distribution of basement membrane antigens at the DEJ: (a) bullous pemphigoïde antigen and (b) Type IV collagen.

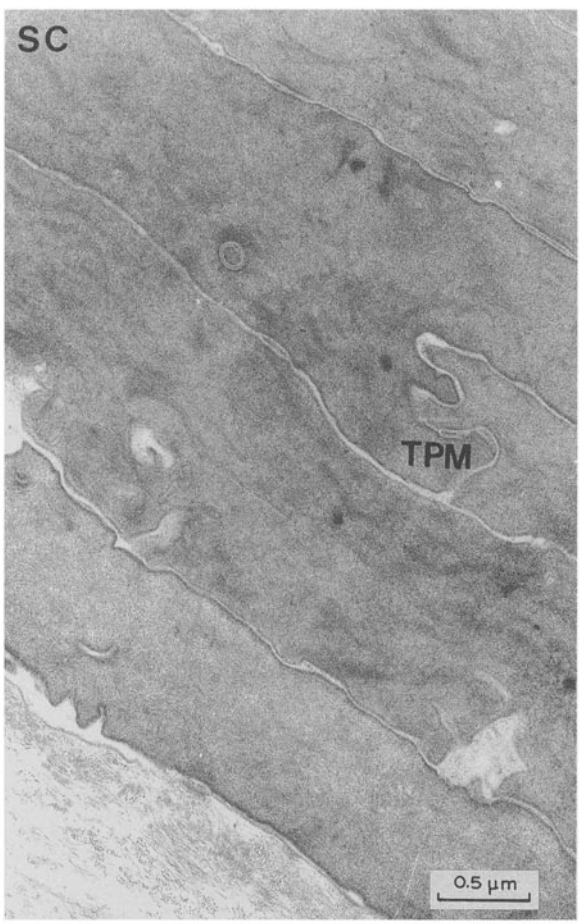

Figure 7 Transmission electron micrograph of the reconstructed stratum corneum (SC) with several layers of cornified anucleate cells filled with tonofilaments. Note the presence of thickened plasma membranes (TPM) characteristic of keratinized cells.

and a polarized distribution at the basal pole of the basal cells (Fig. 8a). The typical linear distribution of Type IV collagen at the DEJ was noted (Fig. 8b).

The distribution of epidermal differentiation markers is shown in Fig. 9: keratins K 1/10 were detected in suprabasal layers of RS (Fig. 9a) and filaggrin was detected in the cytoplasm of cells located in the lower cornified layers and as sparse points in keratohyalin granules in a granular layer (Fig. 9b).

4. Discussion

The dermal substrate (DS) used in this study to reconstruct the skin is cross-linked by chitosane. Chitosane, the polyglucosamine with active free aminogroups, forms an ionic network with acidic groups of collagen and GAGs. This confers a good resistance to the DS and renders it insoluble in physiological liquids. In contrast to the DS cross-linked by chemical agents [6], this DS does not require extensive washing to eliminate toxic residues of the cross-linking agent. The physico-chemical properties, cytocompatibility and biocompatibility of this DS have been reported elsewhere [9–11].

The pore size (at least 80 µm) of the collagen matrix is important for fibroblast ingrowth, as well as for revascularization after transplantation [11]. On the other hand, epidermization of the DS requires a smaller pore size to avoid keratinocyte infiltration. Therefore, keratinocytes were seeded on to the side which was air-exposed during lyophilization and had a smooth surface with very small pores.

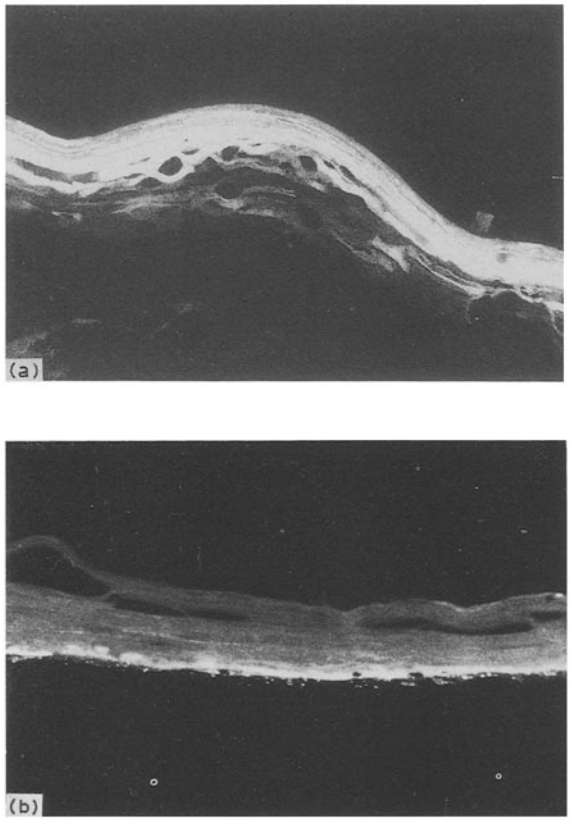

Figure 9 Immunofluorescence studies of RS: distribution of epidermal differentiation markers: (a) keratins 1/10 and (b) filaggrin.

Several reports demonstrate that a collagen matrix containing fibroblasts improves epidermal cell differentiation [12, 13]. It is shown here that NHK cultured on a fibroblast containing DS and exposed to the air, give rise to a reconstructed skin presenting important similarities to natural skin. All epidermal strata are present, including the protective stratum corneum. Processes of epidermal differentiation including expression of filaggrin and keratins, contribute to this similarity.

At the "dermo-epidermal" junction of the RS, the formation of a basement membrane-like structure is noted with the presence of Type IV collagen and the bullous pemphigoïde antigen. Although the epidermal adnexal structures (hair, sebaceous and sweat glands) and immune cells are absent in this skin substitute, this reconstructed epidermis possesses the essential histological compartments of skin. Further analyses of lipid content of the stratum corneum may contribute to understand whether the barrier function and drug metabolism in RS are similar to normal skin.

For grafting purposes, as the epidermis-like tissue reported here is already strongly attached to the DE, the early formation of a complete DEJ is more expectable compared to that of cultured epidermal sheets alone.

In conclusion, reconstructed skin, based on our chitosane cross-linked collagen–GAG, is morphologically very similar to normal human skin and should thus provide a useful tool for *in vitro* toxicological studies, as well as a suitable wound covering for patients suffering extensive burns.

Acknowledgements

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