Development of a human skin equivalent with original mechanical properties

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Abstract:
Different models of reconstructed skin are available but none of them takes into account mechanical properties of human skin. We attempted to construct a dermo-epidermal equivalent maintained under tension on a biomaterial. It consists in 3D mesh, selected among others according to its biocompatibility, bioadhesion properties and mechanical characteristics close to skin *in vivo*. A dermal equivalent (collagen and dermal fibroblasts) is first reconstructed on ring of the biomaterial which inhibits its retraction, allowing the differentiation of fibroblasts into myofibroblasts expressing α-sm actin. The construct is further epidermized with human keratinocytes. The epithelial cells monolayer is placed at the air/liquid interface to allow epidermis differentiation. The immunohistochemical study reveals the presence of a differentiated epidermis similar to skin *in vivo*. To conclude, we report here the construction of a skin substitute maintained under tension, including functionalized cells: myofibroblasts. This original model may be attractive for *in vitro* experiments and for clinical treatments.

Key words: Biomaterial, cutaneous engineering, skin equivalent, mechanical tension, myofibroblasts, keratinocytes.

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Introduction

Skin wound healing is a pluriphasic process which is essential for maintaining the homeostasis of an organism. This phenomenon is classically described in 3 phases: inflammation, proliferation and remodelling. Briefly, after losing the cutaneous substance, an inflammatory and vascular phase occurs, which is followed by the recruitment of fibroblasts at the lesion site. Fibroblasts proliferate and secrete components of the extracellular matrix (ECM), thus forming granulation tissue. Afterwards they differentiate into myofibroblasts rich in α-SM actin and able to develop contractile forces which allow bringing wound edges together. Keratinocytes then colonise the surface of the newly-formed dermis, proliferate to form the different *stratum* of the epidermis. The last phase, remodelling, which corresponds to the maturing of the wound, may last several months *(Clark 1996*). In chronic wounds, such as in leg ulcers, a delay in healing may be observed. The chronicity of the wound, initially related to venous insufficiency, is generally linked to alterations in cellular functions: a decrease in migration capacities and cell proliferation *(Raffetto 2009)*, an alteration in ECM deposition and reorganisation (*Cook 2000*), and cytokines / growth factors secretion *(Wysocki 1993*), as well as a defect in myofibroblastic differentiation. When wound cleansing together with the use of different dressings does not allow wound healing, both graft in pellets and cutaneous substitutes may be used in order to improve healing. Skin substitutes include dermal equivalents (cellularised or not), epidermal equivalents and in a less way dermo-epidermal models *(MacNeil 2007)*. However, none of these substitutes takes into account

natural skin mechanical properties (*Jacquet 2008*). Indeed, mechanical forces developed by myofibroblasts during wound healing are essential for skin wound healing. Reciprocally, the application of mechanical forces on fibroblasts leads to the modification of their phenotype. Indeed, according to the literature, tension forces enhance the synthesis of ECM components, namely collagen type I and III (*Parsons 1999*), and the differentiation into myofibroblasts (*Junker 2008*). The synthesis of proteases, responsible for the degradation of ECM, is down-regulated by tension forces, whereas inhibitors of these proteinases, are up regulated *(Kessler 2001*). So tension forces orientate the physiology of fibroblasts on the side of the deposition of ECM. The resulting scar is stronger compared to healing without mechanical constraint (*Balestrini 2006*). These mechanical properties could be turned to account in order to functionalize cells during skin engineering and also to favour wound healing.

On the basis of this hypothesis, we have developed an autologous cutaneous substitute, the novelty of which being the epidermisation of a dermis kept under tension from the earliest stages of its preparation and until the future graft. Dermal equivalent is reconstructed on a biomaterial selected among others according to its absence of cytotoxicity, bioadhesion properties, thickness, handiness. The present paper will focus on the mechanical and histological features of the skin substitute also obtained.

Materials and methods

Mechanical tests

A DMA (Dynamic Mechanical Analyser) Bose Electroforce 3200 was used to carry out mechanical experiments. A specific "immersion chamber" has been developed for this work. Samples (12 mm of width and 60 mm of length) were subjected to a controlled displacement, at a 0.1 $mm.s^{-1}$ rate. All the experiments were lead at 37°C. Quasi-static tests only allowed the stiffness of the samples to be determined. Some additional harmonic tests were realized to evaluate the damping capacity of materials. In harmonic tests, the sample is subjected to sinusoidal solicitations at a specific frequency. From the load and displacement signals, and particularly thanks to the difference of phase between these two signals (δ), the complex stiffness (K*) can be identified:

$K^* = F^*/X^*$ [1] $K^* = K' + iK''$ [2] $K' = K^* \cos\delta$ and $K'' = K^* \sin\delta$ [3] $\tan\delta = K''/K'$ [4]

with F and X* the dynamic peak-to-peak force and displacement amplitude, K' the storage stiffness, K'' the loss stiffness and tan δ the loss factor.*

The storage stiffness describes the capacity of material to support a load, and so is proportional to the elastic part of the sample. The loss stiffness is the viscous response of the sample and is proportional to the dissipated energy. The loss factor characterises the damping capacity of the material. The same sample geometry than for quasi-static tensile tests is used for harmonic tests. The influence of the temperature, the water content and the frequency of the solicitation on the viscoelastic properties has been investigated.

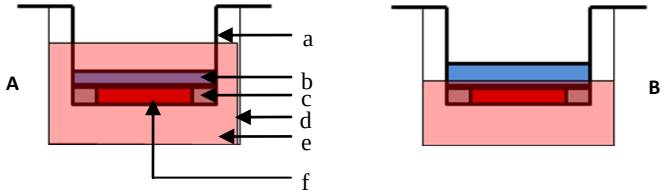
Cutaneous cells culture

Human skin was obtained from abdominal surgery. Human dermal fibroblasts (HDF) were obtained by cell outgrowth from skin explants. HDF were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. Human Epidermal Keratinocytes (HEK) were isolated by Trypsin-EDTA digestion of the epidermis and cultured in Keratinocytes Serum Free Medium supplemented with antibiotics, epidermal growth factor and Bovin pituitary Extract. HDF and HEK were cultured at 37 °C with 5% CO_2 in incubator.

Skin equivalent

Cellularised dermal equivalents composed of collagen type I and dermal fibroblasts were reconstructed using a modified technique developed by Bell *et al.* (*Bell 1979*). The mixture

was poured into insert culture (transwell) in 6-well plate containing a ring of biomaterial and placed in incubator to allow the polymerization of dermal equivalent (1h, 37°C). Just after polymerization, HEK were seeded on the top of dermal equivalent in Green medium and further cultured in immersed condition until obtaining a confluent monolayer (fig 1 A). The culture was then pursued at the air/liquid interface during 14 more days to allow epidermis differentiation (fig 1 B).



Histology and immunohistochemical analysis

Skin equivalents, first fixed in paraformaldehyde and dehydrated were embedded in paraffin. 7 µm sections were stained by hematoxylin/fuschine or hematoxylin/fuschine/light green. The expression of α-sm actin was assessed on fixed lattices by immunostaining.

Results and discussion

Mechanical properties of biomaterial

	Stiffness (N.mm^{-1})	
	Air 37°C	*Water 37°C*
3D	0.093	*0.053*
*Human skin**	*0.025*	

Table I: Stiffness of the selected biomaterial in the axial direction. Comparison of the tests carried out in the water and in the air.
* Equivalent human skin stiffness calculated in considering a Young's modulus of about 1 MPa *(in vivo)*.

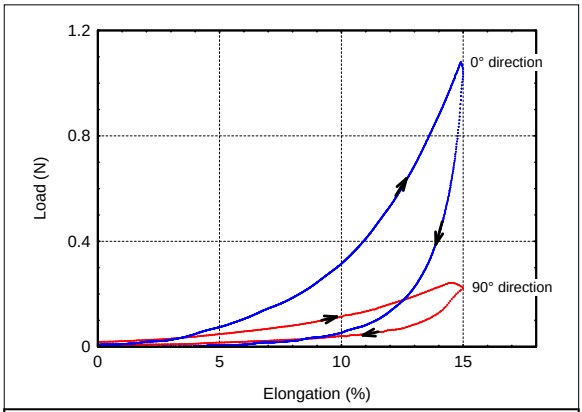


Figure 2: Load – elongation curves of the selected biomaterial according to the material direction. Loading and unloading behaviour are represented.

From the mechanical point of view, the biomaterial intended to be used in the construction of dermal equivalents has to present properties in accordance with *in vivo* human skin which is an inhomogeneous, nonlinear viscoelastic, anisotropic material with a Young's modulus which can be approximated to 1 MPa (*Elsner 2002 and Agache 2004*). So the identified mechanical criteria chosen to select the biomaterials are the rigidity, the deformation and damping capacity, the resistance to water-saturated conditions. Considering the stiffness of the material in water-saturated conditions (Table I) the rigidity of the 3D polyester material is in a good agreement with the one of the human skin. Tensile tests also clearly emphasises the anisotropy of the mechanical properties of the "3D" textile (Figure 2) and in the large strains field, a high non-linearity. As in human skin, the viscoelastic properties of the constitutive polymer of this technical textile, *i.e.* polyester, are affected by temperature and humidity. Harmonic tests carried out in different condition of temperature and humidity (dried or immersed in water) allowed the variation of the viscoelastic properties to be underlined.

Characterization of skin substitute

The architecture and organization of the reconstructed epidermis (fig 3 B and C) are closely parallel to those of human skin *in vivo* (fig 3 A). Indeed, the dermis is surrounded by multiple layers of keratinocytes with flat cells in the most external layer of this reconstructed epidermis comparable to *stratum corneum.*The presence of α-sm actin in the dermal equivalent brings to light the differentiation of fibroblast into myofibroblasts, contractile cells, expressing α-sm

actin, which are essential in cutaneous wound healing (fig 4 C). Dermal fibroblasts adopt this phenotype in response to tension forces created by the presence of the biomaterial which inhibits the natural retraction of dermal construct. So our construct provides functionalized cells for future grafting.

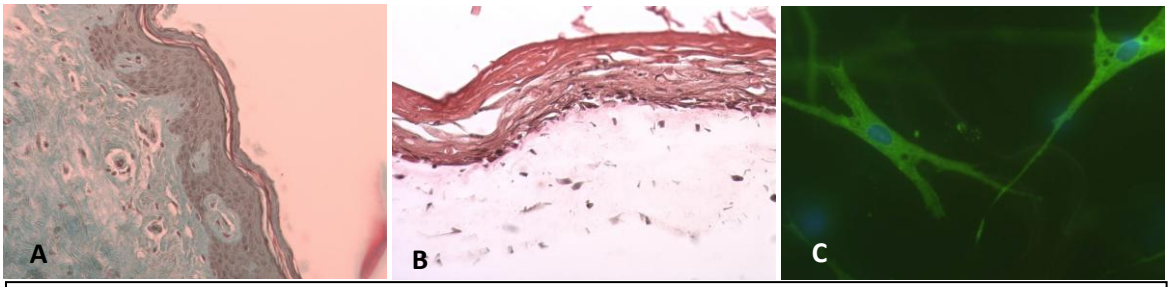


Figure 3: Histological and immunohistochemical analysis of the obtained skin substitute. A: histological aspect of normal human skin (x10). B: histological aspect of reconstructed skin (B: x20). C: Immunostaining of α-sm actin expressed by myofibroblasts (x40).

Conclusion

We reported here an original model of autologous skin substitute with a pluristratified epidermis upon a reconstructed dermis maintained under tension thanks to the presence of biomaterial with mechanical properties similar to those of skin *in vivo*. This skin substitute with functionalized cells, myofibroblasts, may be an interesting tool both for clinical uses, namely in chronic wounds, and as *in vitro* model for toxicological/pharmacological research.

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