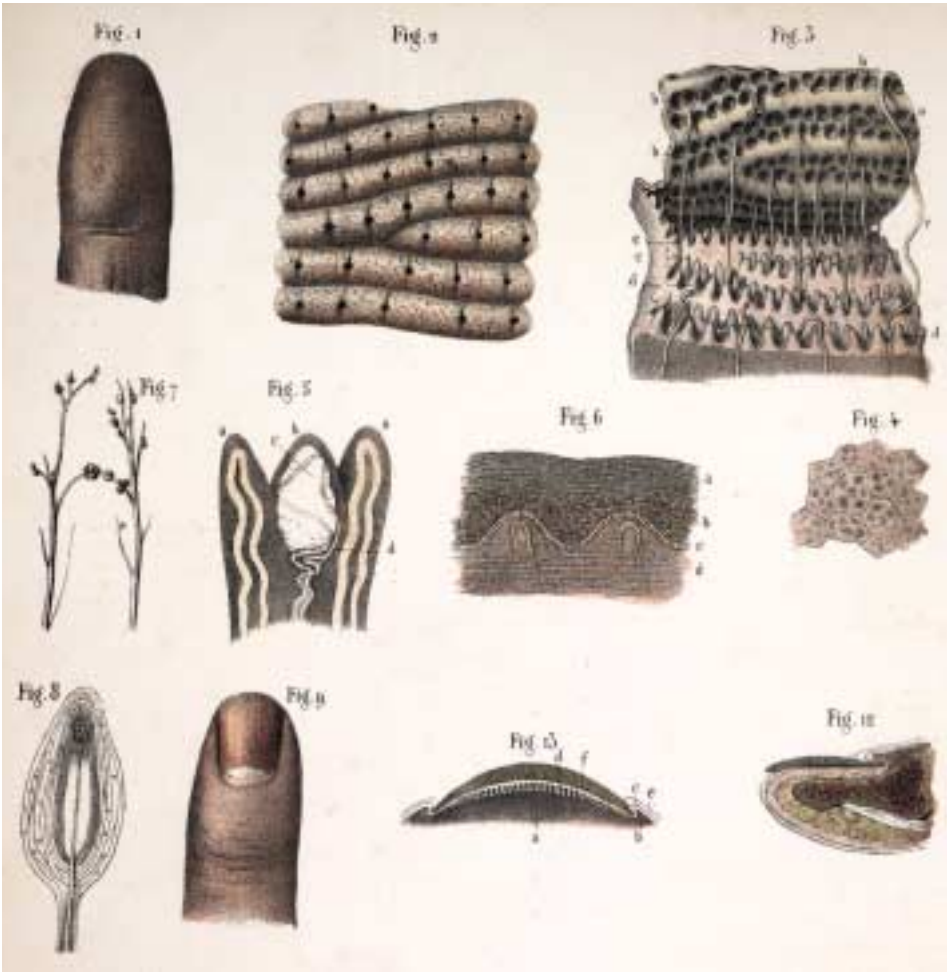
L'ORÉAL

Rebuilding the Skin



POUR LA SCIENCE

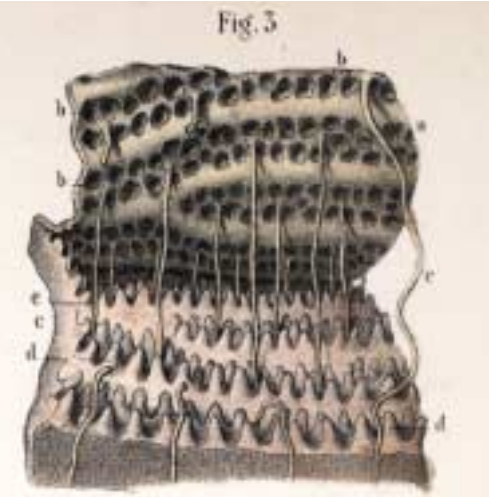
L'ORÉAL

Direction Générale
Recherche et Développement

www.loreal.com

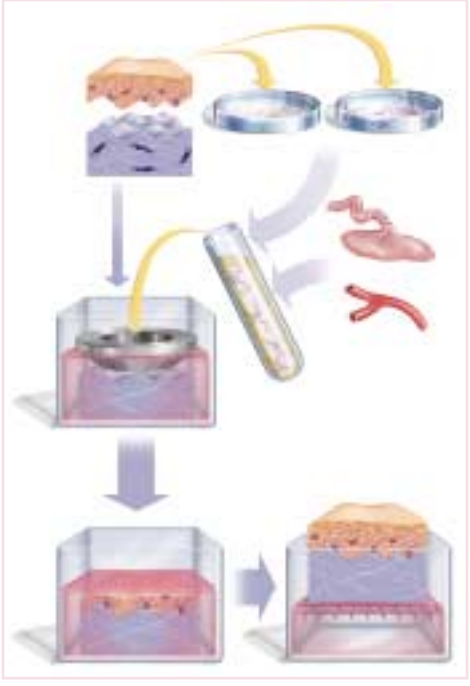
L'ORÉAL

Rebuilding the Skin



POUR LA SCIENCE

REBUILDING THE SKIN



First operationnal microscope invented by Leeuwenwhoek (1632-1723). With this apparatus, he could observe and draw “animalcules” (microbes), different types of cells, such as red cells and spermatozoids.

THE SKIN REVEALED

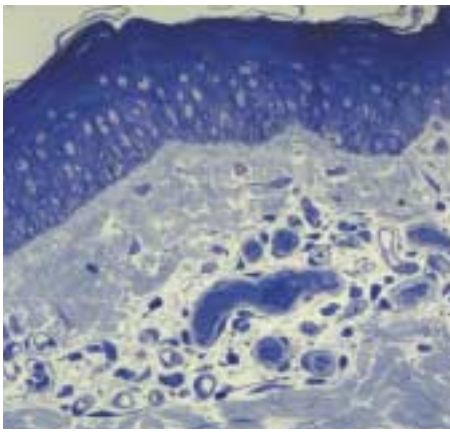
"The skin is as perfect as it is useful and nature has been shown to be as beneficent as it is inexhaustible." The ideas of the dermatologist Jean-Louis Alibert on the structure of the skin reflect rather well those of the beginning of the XIXth century. In fact, it was the appearance of the first achromatic lenses in the 1830s, one of the most important technological innovations of the first half of the XIXth century, which put an end to philosophical speculations and caused unprecedented interest in more detailed study of the organs in general and the skin in particular.

While the use of the first microscopes by Malpighi dates from the XVIIth century, their rudimentary character only allowed rather gross observations. Numerous works on tissue and cell structure appeared, led by scientists such as Vichow, Pacini and Purkinje. As early as 1855, Breshet saw the skin "not as a simple membrane made of more or less numerous layers but rather as an assembly of organs with distinct functions, held together by a dense tissue, with nerves, blood and lymphatic vessels running through it". These observations stimulated a new approach to skin science with the area of study changing from macro-anatomy to micro-anatomy. This new perception revolutionised skin physiology and the first human epidermal grafts which were made in 1862 and 1872 by Reverdin and Ollier initiated studies on immunology and the problems of homografts in general. A little later, Wolfe and Krause introduced grafts of whole skin, this practice and Ollier’s technique remaining the two

types of grafts in widespread use for a long time. Thus, it was only through large-scale skin destruction such as burns that the skin was consciously seen to play a vital role.

While it occupies a privileged position, its role in the recognition of foreign bodies was ignored for a long time and its barrier function seemed to be so obvious that it was almost forgotten. It was only in the middle of the XXth century that the physiological importance of the skin was clearly revealed : the study of its immunological functions, its cell types, its primordial role in protecting from external attack revealed the skin as a real organ. While it has been the subject of innumerable attempts at *in vitro* culture for nearly a century, this new knowledge along with great progress in cell culture techniques was to reward scientists' efforts.

Since 1979, the year of the first reconstructed epidermis, numerous models have been developed to reconstruct *in vitro*, either an epidermis on an inert or synthetic support, the barrier function of which is close to that of normal skin, or a dermo-epidermal structure or human skin. They form tools for the evaluation and toxicology in cosmetic industry and for experimental dermatology. They are also used by scientists to provide answers to certain basic questions : How do cells communicate with each other ? How do skin cancers develop ? How does the sun cause so much damage ? How does skin age ?.. More and more sophisticated models are developped and thus provide a direct application for tissue engineering : restoring the skin of major burns and ulceration.



More and more advanced and more suitable techniques have been developed during the XX^{e} century and have revealed to scientists the complex architecture of the skin. Under optical microscopy, staining with toluidine blue reveals the epidermis and the dermis.

Seen by scanning electron microscopy (SEM), the architecture of the skin is characterised by its many strata ; the outer layer of cells, the horny layer, being a barrier against external aggression.

THE ARCHITECTURE OF THE SKIN

The skin is also related to the *integument*, which is used for seeds etc. and refers to the outer covering. The skin is the biggest and heaviest organ since it represents a surface area of nearly 2 m^2 and weighs up to 4 kg in a 75 kg adult. Its complexity is reflected in its architecture which is formed of three compartments, three different superimposed tissues : the epidermis, the most external ; the dermis, and the deepest the hypodermis.

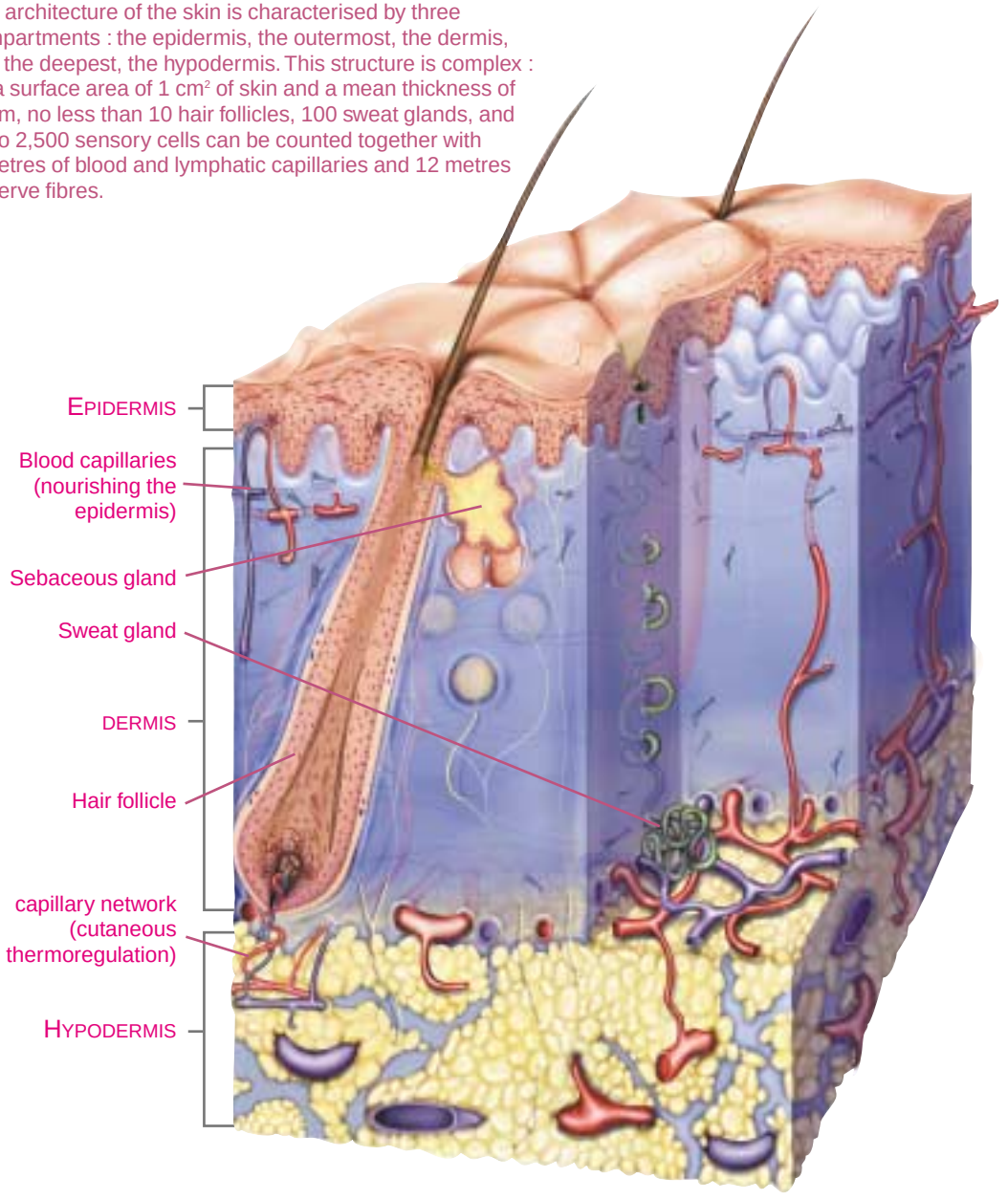
A protective envelope

The epidermis (from the Greek *epi*, above and *derma*, the skin) forms the external structure of the skin. Thin, 0.05 mm, on the eyelids, though 1.5 mm thick on the soles of the feet, it nevertheless provides protection for the organism, in particular through the presence of the *stratum corneum* or horny layer.

The outermost layer of the epidermis, forms a barrier against attacks from the environment, infectious agents and any substance with which the skin may come into contact. It is the ultimate result of a 4 to 6 week journey by the keratinocytes during which these cells, the most numerous in the epidermis, divide and undergo a process of differentiation

accompanied by structural and biochemical modifications. These keratinocytes are distributed in four superimposed layers, which explains the stratified character of the epidermis : the basal layer (or mitotic layer), the spinous layer (also known as the Malphigian layer), the granular layer and the horny layer.

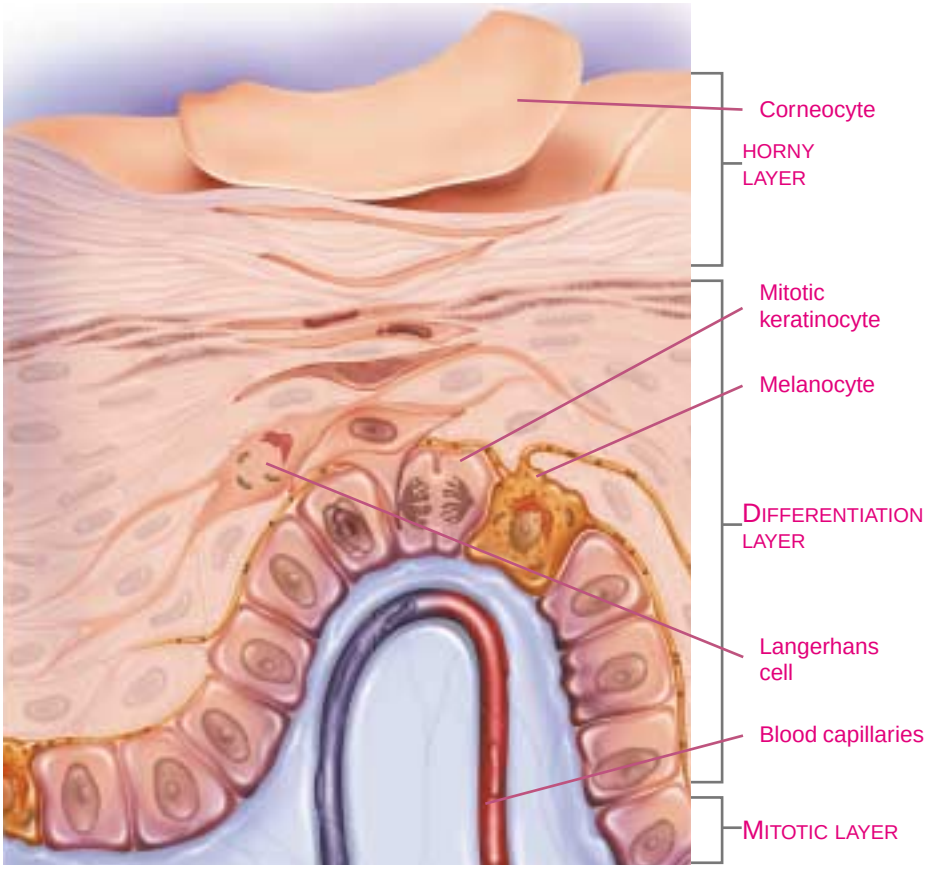
The architecture of the skin is characterised by three compartments : the epidermis, the outermost, the dermis, and the deepest, the hypodermis. This structure is complex : for a surface area of 1 cm^2 of skin and a mean thickness of 3 mm, no less than 10 hair follicles, 100 sweat glands, and up to 2,500 sensory cells can be counted together with 3 metres of blood and lymphatic capillaries and 12 metres of nerve fibres.



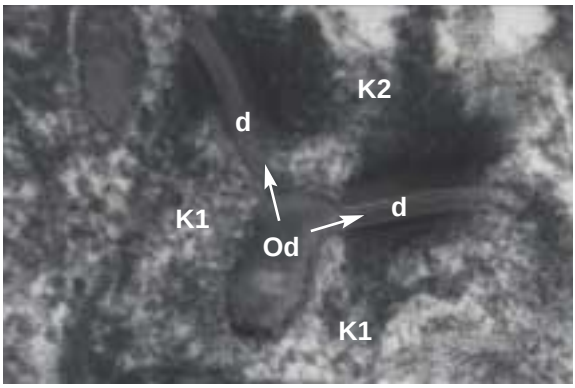
The keratinocytes' journey

The journey begins in the basal layer, the deepest part of the epidermis, which is formed from a single row of keratinocytes linked by structures like press studs, desmosomes, and to the subjacent dermis by hemi-desmosomes. In this layer the keratinocytes divide : each cell gives rise to two identical daughter cells. One of the two migrates towards the upper layer while the other enters a new process of cell division to give rise to two more daughter cells and so on. While moving away from the basal layer the keratinocytes begin to differentiate : they change shape,

RENEWAL OF THE EPIDERMIS



From bottom to top : The keratinocytes divide in the skin mitotic layer. Each keratinocyte divides to produce two identical daughter cells. One remains in the mitotic layer in order to divide again whereas the other migrates to the upper layer, the differentiation layer, where it will undergo numerous morphological and biochemical changes. In the horny layer, keratinocytes, now called corneocytes, are anucleate flattened cells, filled with keratins. Under the action of specific enzymes, corneocytes lose their cohesion and slough off the surface in a process known as desquamation.

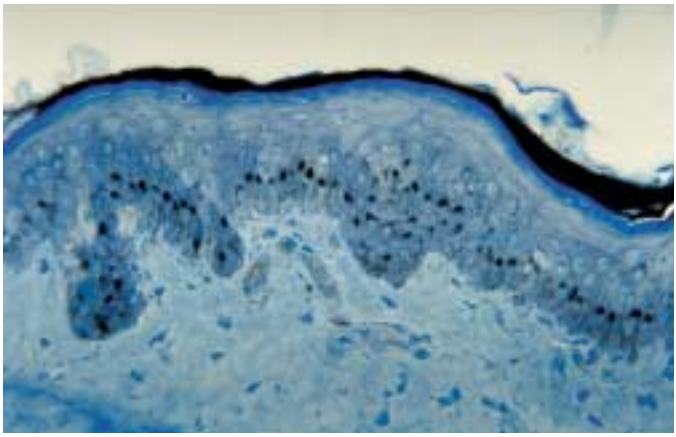


In the differentiation layer, two keratinocytes (K1 and K2) are linked by desmosomes (d) visible by transmission electron microscopy. An Odland body (Od) where the lipids are synthesised migrates to liberate its contents into the intercellular space *(arrows)*.

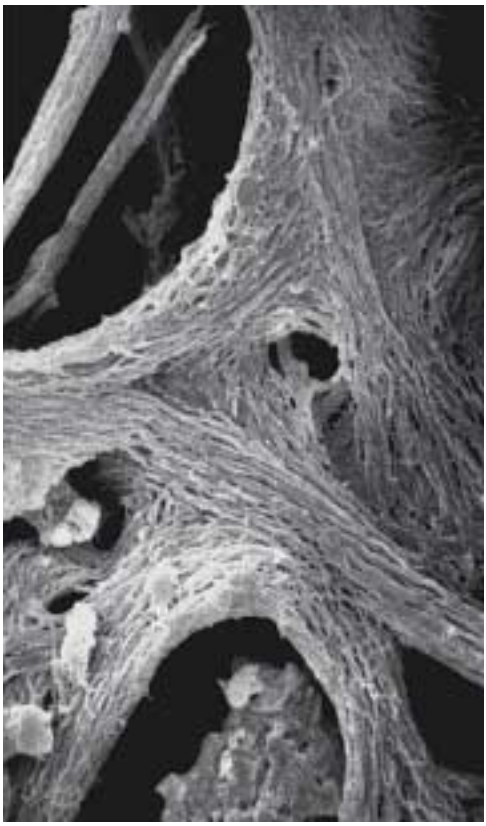
progressively flattening and finaly become corneocytes. These flat non-nucleate, keratin filled cells are linked by corneodesmosomes which ensure compact stacking of the cells. The cohesion and flexibility of the structure is strengthened by a wide variety of lipids (ceramides, cholesterol, fatty acids) in the extra-cellular environment arranged between the cells. At the surface of the skin, the corneocytes lose this adhesion through the action of a specific enzyme which detaches them one after the other in an imperceptible way in normal skin : this is the phenomenon of desquamation.

Cells to colour, others to defend

Other cells are located in the basal layer of the epidermis : melanocytes. Fourty times less numerous than keratinocytes, they are in part responsible for skin pigmentation. They synthesise melanin pigments in the form of little grains, called melanosomes. Melanocytes are recognised by their long processes, called dendrites, where the melanosomes are



Histological section of an African skin showing the melanosomes (black spots) which, once transferred to keratinocytes, form a helmet over the nucleus of these cells to protect the DNA.



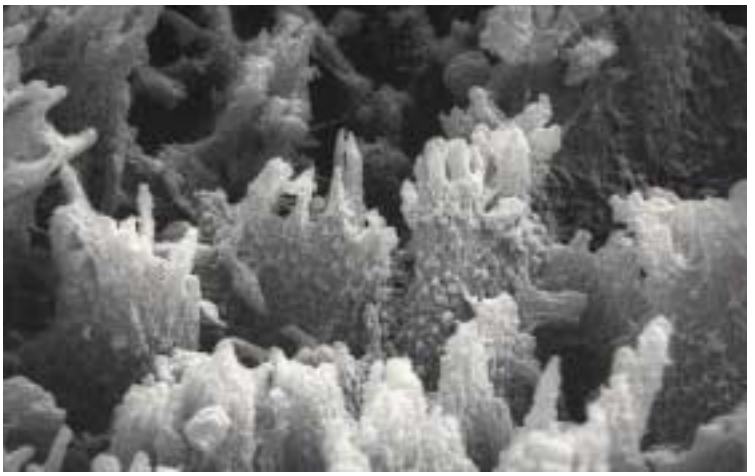
The dermis is formed from a matrix of protein fibres : collagen microfibrils *(above)* and elastin fibres *(below)*. Together, bathed in a gel, they give the dermis is properties of strength and elasticity.

concentrated before being transferred to neighbouring keratinocytes. A melanocyte can thus supply 40 keratinocytes with melanin which, during their migration to the surface gradually digest them. This collaboration between the two cell types results in the spread of pigments and contributes to giving the epidermis its coloured appearance.

Discovered in 1868 by a young Berlin doctor, Paul Langerhans, the cells of the same name (2-5 % of the epidermis) are derived from haemopoietic stem cells in the bone marrow and occur in the differentiation layer. Langerhans cells are also dendritic cells and form essential elements of the organism's defence mechanism. Responsible for detecting foreign agents (antigens) which have penetrated the epidermis, these cells capture the intruder and then move to the lymphatic ganglia in the dermis, where they transfer it to the lymphocytes. Due to a cellular type of immune response, the antigen is neutralised then eliminated.

The dermis, a support tissue

Much thicker than the epidermis, the dermis (1 to 4 mm) is the solid support for the skin. It confers on it its great strength and



Dermo-epidermal junction is an area of exchange between the two compartments of the skin. This scanning electron micrograph reveals the epidermal crests.

elasticity when subjected to all sorts of tension, traction and torsion : so many different strains that it absorbs mainly through a matrix of fibres produced by the fibroblasts, the main cells of the dermis. The fibroblasts are essentially located in the papillary dermis close to the epidermis, and are poorly represented in the deeper dermis or reticular dermis. They are specialised for the synthesis of two types of protein fibres : collagen fibres and elastin fibres which make up the extracellular matrix. The first constitute 70 % of the proteins of the dermis and provide it with its resistance to tension and traction, while the second give it its elastic properties. This network of fibres is bathed in a gel composed of macromolecules (glycoproteins, GAG or glycoaminoglycans) which trap water and contribute to maintain the cohesion and structure of the dermis. With its connective tissue properties, it is the dermis which gives the skin its firmness and tone.

The absence of blood vessels in the epidermis means that the nutrients and energy essential for the cells of the basal layer are provided from the dermis. Exchange and nutritive irrigation occurs particularly through the capillary network at the junction between the dermis and the epidermis.

In young skin, the dermo-epidermal junction has the appearance of a mountain range and is called the "dermal papillae". On the epidermal side, this structure allows the anchoring of the epidermal keratinocytes to the papillary dermis and on the dermal side the anchoring fibres interact with the basal membrane forming a network which traps collagen fibres. Among other things, its biochemical composition

is characterised by the presence of collagen IV and laminin, two structural elements involved in cellular attachment and which provide the architecture of the basement membrane.

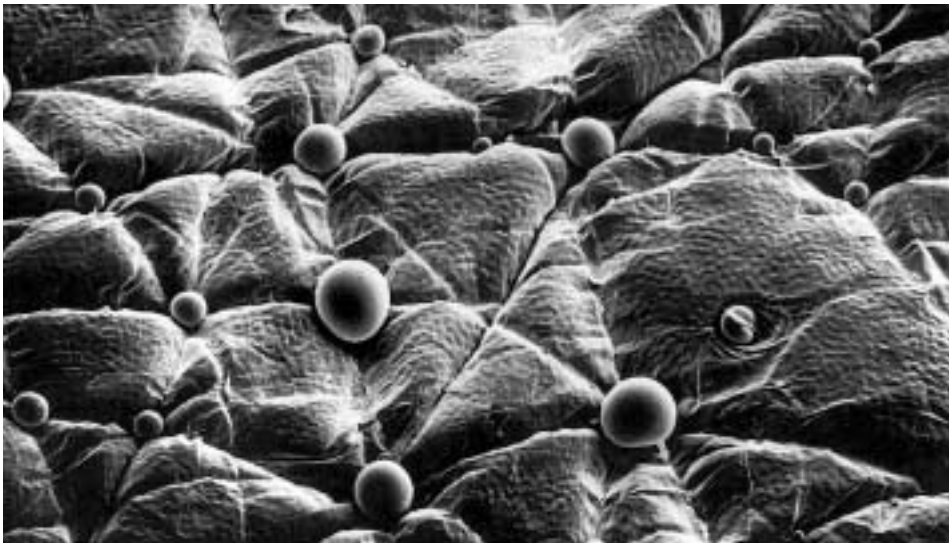
The capillaries are joined to a vascular network located deeper in the dermis which plays an important role in thermoregulation and is involved in the defence mechanisms of the organism and in the wound healing process.

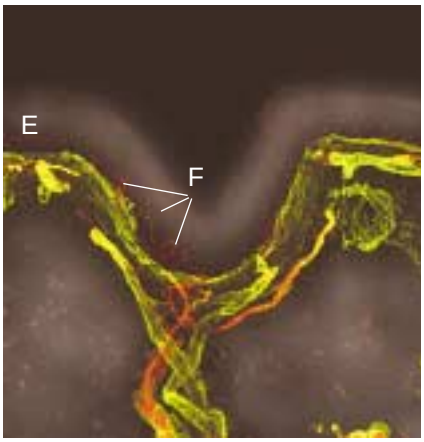
An energy reserve

The hypodermis is the innermost and thickest layer of the skin. It invaginates into the dermis and is attached to the latter, immediately above it, by collagen and elastin fibres. It is essentially composed of a type of cells specialised in accumulating and storing fats, the adipocytes. It is the organism's energy reserve.

The skin also has appendages : hair, eye-lashes, eye brows and nails, which are prolongations of the epidermis rooted in the dermis, or even in the hypodermis and eccrine and apocrine sweat glands and sebaceous glands, which respectively play a part in thermoregulation, the secretion of pheromones and sebum. The main role of these glands is therefore to cool the organism and to synthesis substances which will, depending on their nature, protect or make the skin more flexible, lubricate the hair, eliminate mineral elements, cholesterol etc.

Under scanning electron microscopy, the corneocytes are arranged like cobblestones and drops of sweat secreted by the eccrine glands are excreted through the skin pores.





The free nerve endings (F) reach as far as the epidermis. Some of them are intimately associated with the Merkel cells, which are involved in tactile perception.

The listening skin

The skin is the site of perception ; it acts as the vehicle for the sensations of touch, light touch, contact, pressure, temperature, dryness and pain. These events are perceived by the skin as external information which will be translated, transformed and processed by the cutaneous neuro-sensory system which includes a network of free nerve endings and receptors. Skin receptors take the form of corpuscles (Meissner, Pacini, Krause and Ruffini corpuscles) or free nerve endings, a number of which associated with Merkel cells, go as far up as the epidermis. They receive tactile information (from vibration to pressure) which is passed via the network of nerve fibres as far as the brain in the form of nervous input. Messages are formed which result in a sensation.

The skin is thus so complex that the difficulties and extraordinary scientific challenge of reconstructing it in the laboratory can well be imagined. This biotechnological advance is the result of several decades of research the main features of which are touched on in what follows.

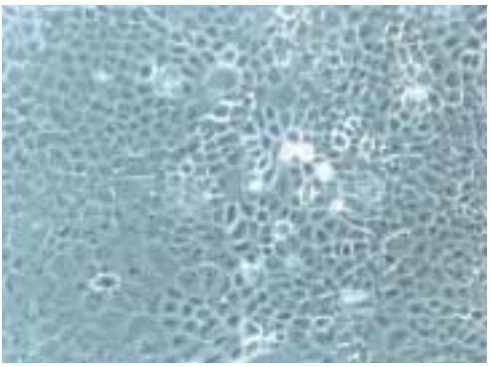
In 1975, J.-G. Rheinwald and H. Green cultivated keratinocytes in series on a nutrient layer. They obtained in the form of fragments the first epidermal substitute consisting of several layers of keratinocytes.

THE ADVENT OF TISSUE ENGINEERING

Tissue engineering is a life science which aims at developing biological substitutes for restoring, maintaining or improving a tissue function. As far as the skin is concerned, it consists of reconstructed skin models which include some functions of the epidermis and / or those of the dermis. This approach consists of associating cells and natural or synthetic biodegradable polymers and is today going through a stage of rapid and spectacular development. Historically, skin engineering has benefited from the great progress made in cell culture techniques, a first key step in the development of reconstructed skin models. The first culture technique for keratinocytes consisted in the 1960s of placing a 2 mm^2 fragment of skin in a Petri dish with the dermal side facing downwards. These explants were left open to the air for a few minutes to make them adhere to the support before being immersed in a culture medium. The keratinocytes migrated to form a crown of cells while the explant, which became disorganised with time, was eliminated. After ten days, 2 or 3 layers of superimposed keratinocytes showing signs of incomplete differentiation were obtained.

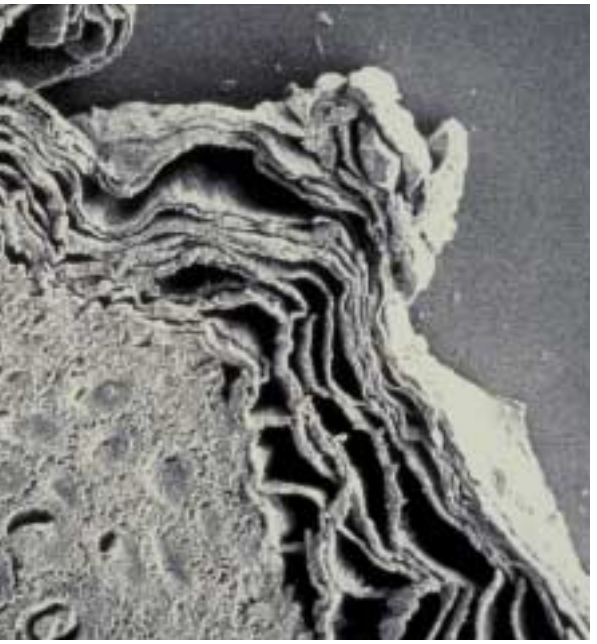
While explant cultures are still used for toxicology and cytotoxicity trials, the main disadvantage of this technique is due to the presence of fibroblasts from the dermis of the explant : they proliferate at the same time as the keratinocytes and can completely invade the culture of these cells.

More and more specialised culture media were necessary to obtain a skin substitute with the characteristics of human skin. Though at the end of the 1970s, only the keratinocytes had been successfully cultured, the development of culture methods was to be the key towards the co-culture of different types of epidermal cells.



A big step forward was achieved in 1975. J.-G. Rheinwald and H. Green succeeded in cultivating keratinocytes in series over a layer of nutrient providing fibroblasts, 3T3 cells. The latter are unable to multiply but retain their property of secreting a large number of proteins, such as laminin and growth factors, thus encouraging the attachment and multiplication of keratinocytes. Seeded first of all at low density on this nutritive layer the keratinocytes will cover the whole surface or reach confluency. Occurring in the form of fragments, this first epidermal substitute consisted of several layers of human keratinocytes in culture. It was not yet a truly reconstructed skin : the fragments had no connective tissue support (dermal equivalent) and the permanent immersion of the cultures prevented complete epidermal differentiation, hence the absence of the horny layer.

More physiological culture conditions were therefore required to obtain a reconstructed skin including differentiated cell layers with human skin characteristics. It was with this aim and the idea of using three-dimensional cultures that skin engineering was to develop. Since J.-G. Rheinwald and H. Green, all models developed and subsequently described have had a culture support, or dermal equivalent, provided for the keratinocytes and the exposure of the culture at the air-liquid interface. The only difference between these models is in the types of cells included and in the composition and production of the supports.



The biological machinery produced by the epidermis has the main function of producing the horny layer. This layer is in direct contact with the external environment and therefore plays a vital role as a barrier against all sorts of aggression. The first objective of research workers was therefore the reconstruction of an epidermis with a horny layer close to that of human skin.

THE RECONSTRUCTED EPIDERMIS ADVENTURE

The importance of the superficial layer of the epidermis was the reason for the development of the first reconstructed epidermis model in which the differentiated horny layer carried out its primordial role as a barrier. In these models, the support was an inert dermis onto which human keratinocytes were seeded which proliferated in immersed culture. A final step of exposure of the culture to the air resulted in the differentiation of the keratinocytes to form the horny layer.

The very first reconstructed epidermis

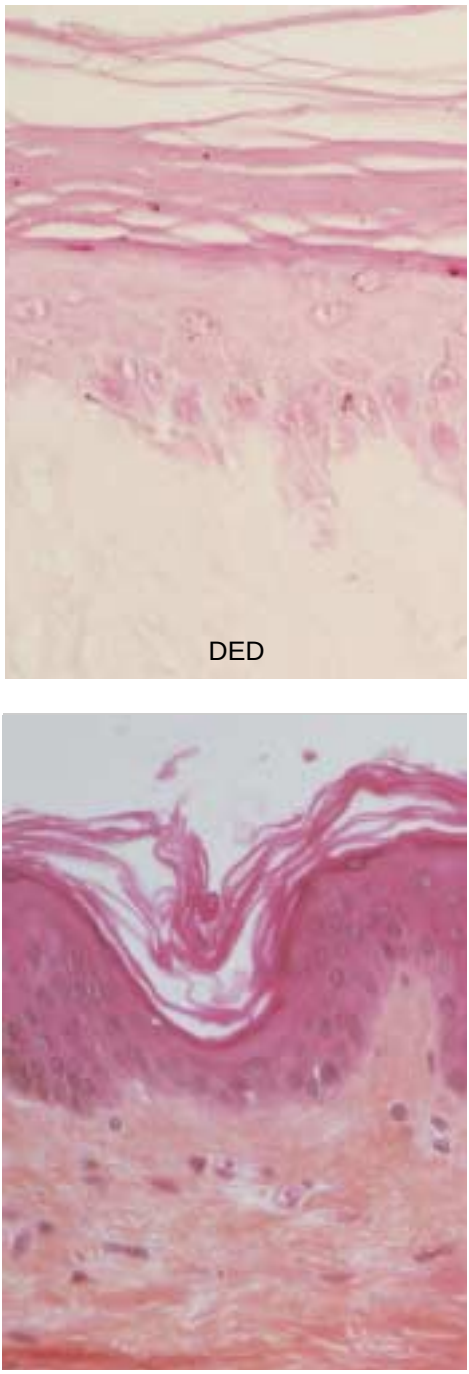
The first model of this type, the Prunieras-Régnier model, was perfected in 1979. A skin biopsy was taken from aesthetic surgery and the epidermis was separated from the dermis by the action of proteases. The dermal fibroblasts were then killed by successive cycles of freezing and defrosting. This inert de-epidermised dermis (DED) was laid flat and the isolated keratinocytes from the epidermis were applied to its surface. This model has several useful features : the DED retains its basal membrane where collagen IV, laminin and GAGs occur, which encourages optimal

reconstruction of a structured differentiated epidermis topped by a horny layer. In addition, the inert dermis forms a natural support for the keratinocytes with a filtering function for nutrient compounds close to that of a normal dermis. It is also in this model that the lipid composition of the horny layer most nearly approaches that observed *in vivo*, with the expression of several classes of ceramides.

Michel Prunieras and Marcelle Régnier joined the L'Oréal group in 1983 where they continued their work. Within L'Oréal laboratories, a research momentum directed at finding new reconstructed epidermis models was to bear fruit. Research workers incorporated melanocyte into the first model…. Then Langerhans cells, thus creating a model possessing the three fundamental cell types of a living epidermis.

A model which tans

The inclusion of melanocytes into these reconstructed models has considerably widened their field of application. It was in the L'Oréal laboratories that R. Schmidt and M. Régnier thus cultured melanocytes extracted from human epidermis. Mixed with keratinocytes in a ratio identical to that found in the normal epidermis (1 melanocyte to 40 keratinocytes),

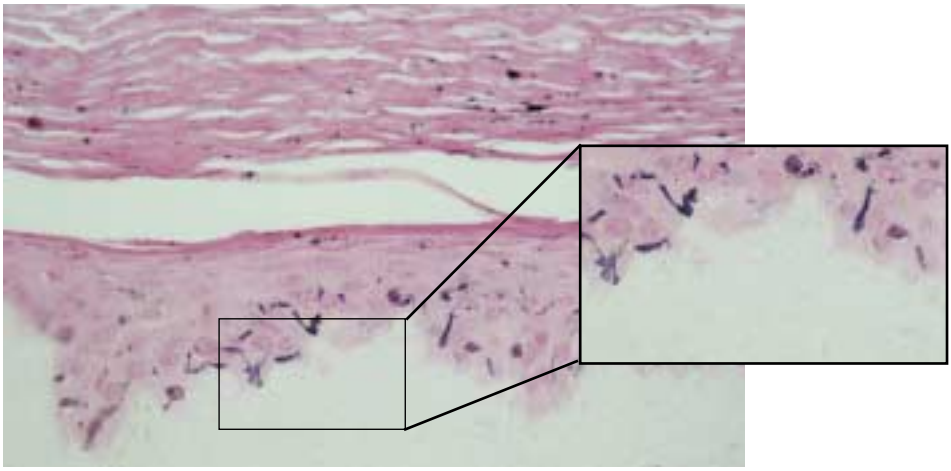


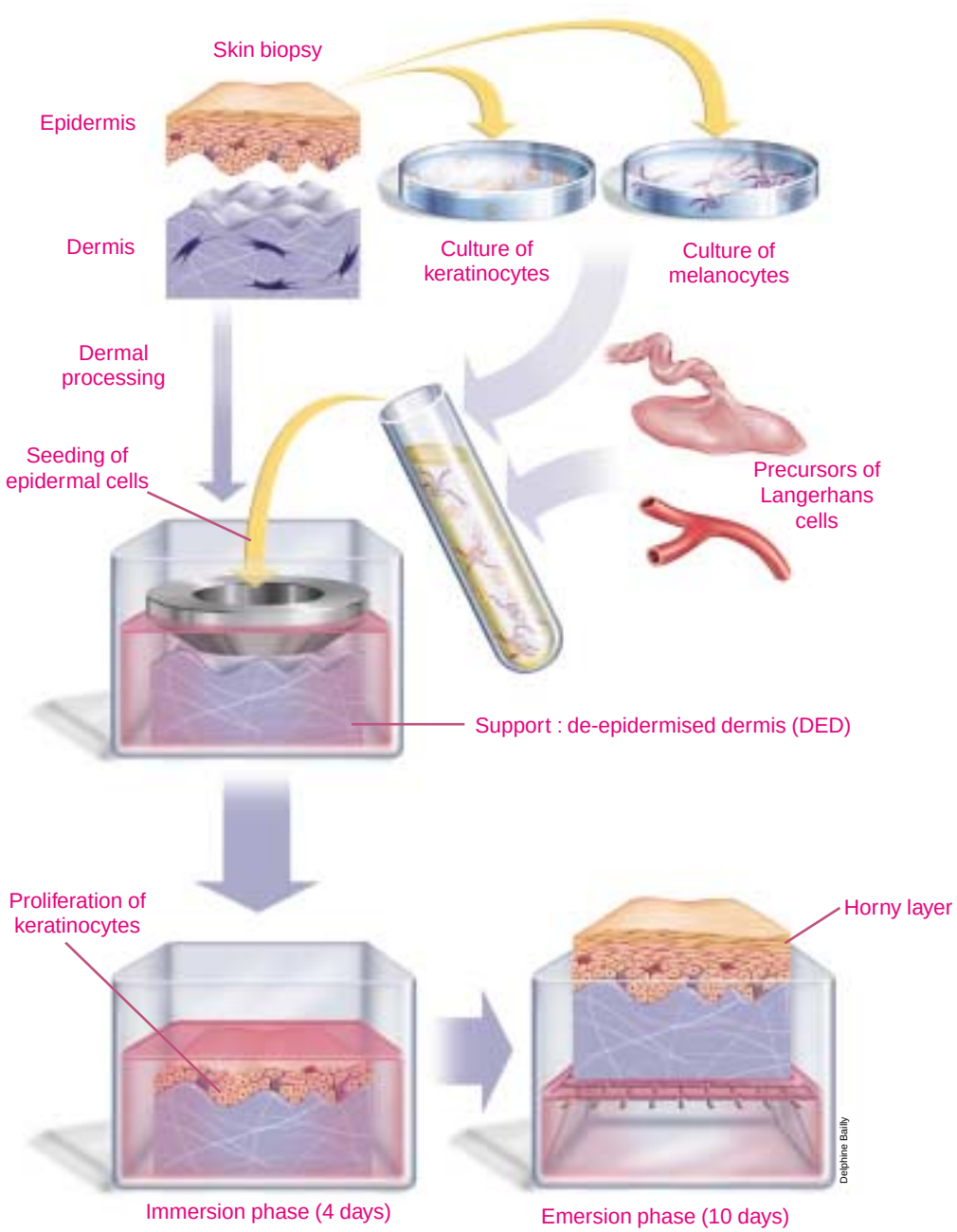
The Prunieras-Régnier model *(above)* was the first reconstructed epidermis on an inert de-epidermised dermis (DED). It has a horny layer close to that of normal human skin *(below)*.

the mixture of cells was seeded onto the de-epidermised dermal support (DED). After 4 days of immersed culture, the DED is kept for 10 days on a grid at the air-liquid interface (emersion phase) to cause differentiation of the keratinocytes. A reconstructed pigmented epidermis with a horny layer is thus formed. In this model, the melanocytes are located in the basal layer, as they are *in vivo*, and can transfer their pigment to neighbouring keratinocytes. Pigmented reconstructed epidermis models are the tools of choice for studying the modulation of melanogenesis by pigmenting and depigmenting agents and for understanding the interactions between keratinocytes and melanocytes, and in particular, the role of the latter in this relationship. They allow the effects of ultraviolet radiation on the epidermis to be estimated and to estimate protection after the topical application of sunscreens such as Mexoryl® SX and XL, two sunscreens developed by L'Oréal chemists. This type of model also makes it possible to study skin phototypes and to see differences, in particular concerning the rate of synthesis of the pigments. Research workers have thus included melanocytes from african, caucasian and asiatic skin.

In collaboration with INSERM, a model of melanoma has been reconstructed by including melanocyte from a malignant source. The model has made it possible to monitor the formation of tumour cells and their migration from the basal layer into the dermis.

M. Régnier and R. Schmidt incorporated melanocytes (in black) in the M. Prunieras model. They thus created a pigmented reconstructed epidermis which today is the method of choice for studying the modulation of melanogenesis or for testing the efficacy of sunscreens.

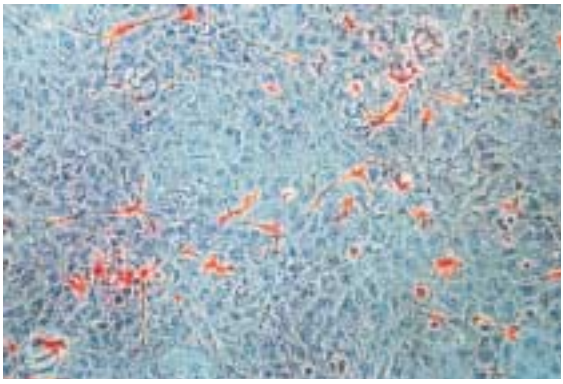




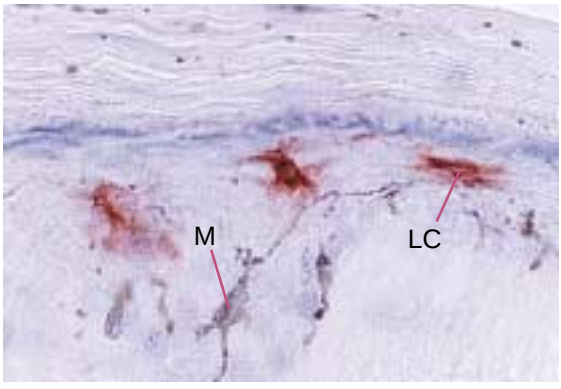
In the epidermis models developed by L'Oréal laboratories, the support is a de-epidermised dermis (DED) into which the epidermal cells are seeded. After a 4-day immersion phase, an emersion phase of 10 days results in the keratinocytes differentiation to form the horny layer. M. Régnier and R. Schmidt achieved integration of Langerhans cells into this epidermis : after co-culture of keratinocytes, melanocytes and precursors of Langerhans cells ($CD34^+$), a model with the three principal types of epidermal cell was created.

A model on the look-out

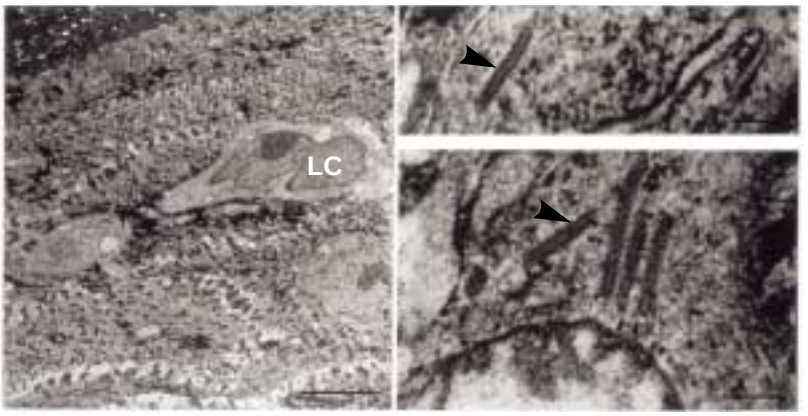
The inclusion of Langerhans cells in a reconstructed epidermis remained a real challenge because the cells slowly divide *in vivo* and do not multiply at all *in vitro*. In 1992, the discovery in umbilical cord blood and in the peripheral blood of adults of Langerhans cell precursors and the possibility of making them multiply *in vitro* removed this obstacle. In 1996, within a European programme, M. Régnier and R. Schmidt in collaboration with INSERM, the Karolinska Institute in Stockholm and the University of Bonn, used the $CD34^+$ haemopoietic precursor derived from the blood. By placing these Langerhans cell precursors in a given culture medium with the melanocytes and keratinocytes, and after seeding these cells onto the DED support, the research workers then succeeded in constructing a model which included the three cell types found in a normal epidermis. As *in vivo*, the melanocytes are to be found in the basal layer and the Langerhans cells in the suprabasal layers. In addition, the Langerhans express the major histocompatibility complex, the CD1a antigen and contain Birbeck granules (rod shaped organelles the function of which remains to be determined).



The production of Langerhans cells *in vitro* was possible by the haemopoietic precursors culture of these cells, $CD34^+$ cells. After 12 to 14 days of culture with keratinocytes, a population of dendritic cells appeared having the characteristics of Langerhans cells, as shown by the red labelling with anti-Lag antibodies.



After having succeeded in producing Langerhans cells *in vitro*, L'Oréal scientists constructed a pigmented immunosensitive epidermis with a horny layer, an important technological advance since it reproduces *in vitro* an epidermis very close to natural tissue. Langerhans cells (LC), melanocyte (M).



In immunosensitive models of the epidermis, the Langerhans cells (LC, *left)* are localised in the suprabasal layer and an enlargement *(right)* shows the Birbeck granules, characteristic of these cells.

Exposed to solar radiation, the morphology of these cells changes, they decrease in number and release cytokines, thus reproducing the phenomenon of photo-immunosuppression, which could be studied with this model. Langerhans cells respond to control substances which provoke allergies, so the use of this model can be imagined for understanding the role of Langerhans cells in the immune response. If it was produced on an industrial scale, this model could also be used for detecting the allergic properties of certain compounds while avoiding the animal skin tests.

Beyond these applications, the study of the complex interactive mechanisms between the cells of the epidermis becomes possible with this model.

Episkin™ is a model of the epidermis marketed in the form of a kit of 12 wells which has been validated for corrosion tests. It is at present undergoing validation for the skin irritation tests.

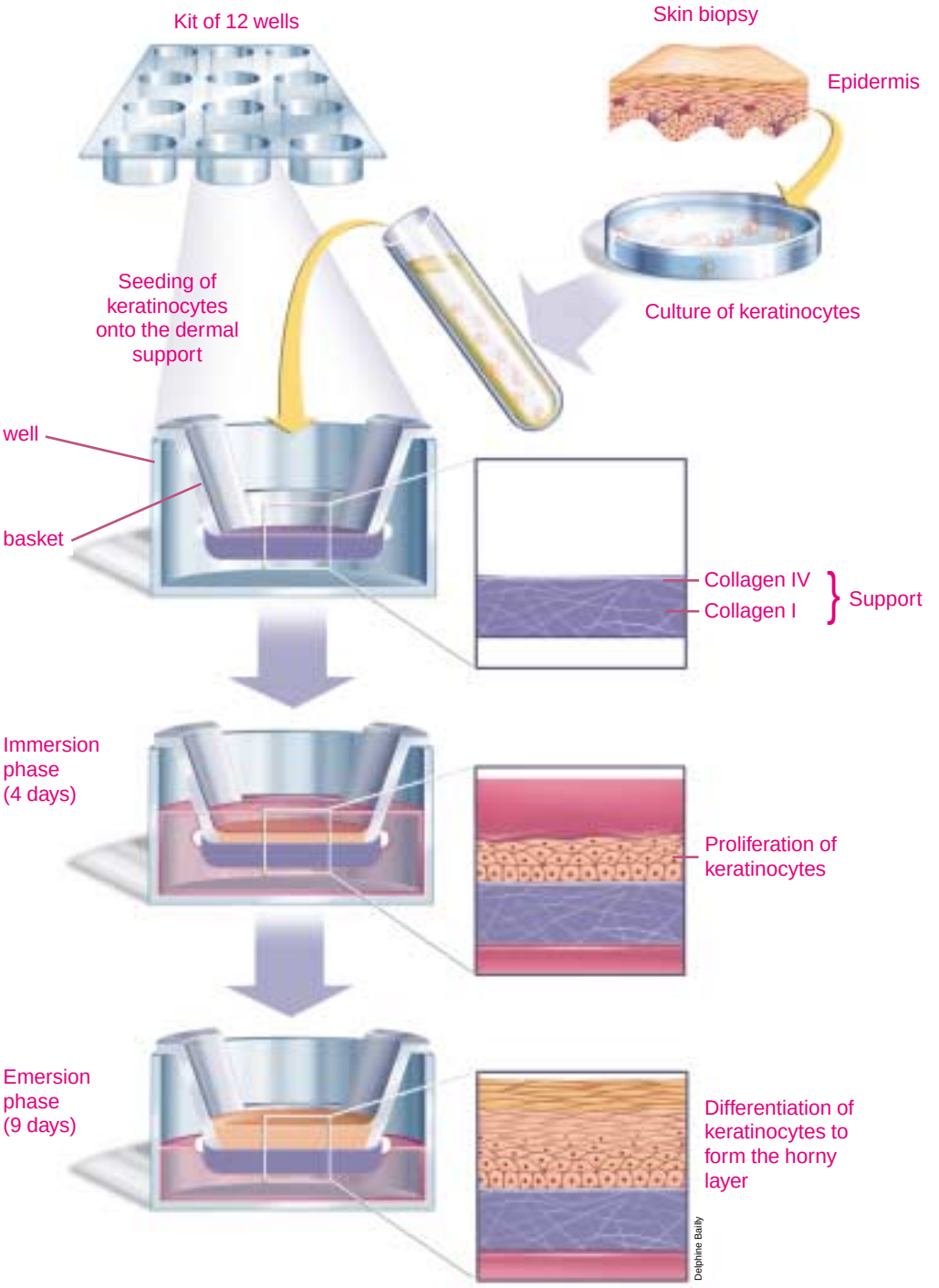
EPISKIN™, A MARKETED MODEL

The development and validation of alternative methods to animal experimentation represents one of the priorities of the cosmetics industry. These methods must predict the effects of cosmetics under their normal conditions of use. Since 1989, the L'Oréal group has no longer tested any finished product on animals and reconstructed epidermis technology is a key element in keeping to this commitment.

Models made on an inert support (the DED) have the qualities required as research tools but samples of human dermis are limited in quantity and subject to wide variations (individual to individual, body regions). These limits exclude the standardisation of this type of model from its large-scale production. In this context, a reconstructed human epidermis model, Episkin™, has been produced on an industrial scale, subject to strict quality controls.

The Episkin™ model

The human reconstructed epidermis developed by E. Tinnois and marketed in the form of a kit by the Imedex Company under the name of Episkin™ was bought by L'Oréal in April 1997. It consists of a dermal support formed from collagens I and IV. Keratinocytes, deposited on this support, divide for 4 days in the culture medium, and then



In each well of the Episkin™ kit, a collagen I gel topped by a layer of collagen IV is placed in a basket. The keratinocytes are seeded onto this dermal support. A 4-day immersion phase induces proliferation of the keratinocytes and an emersion phase of 9 days results in the formation of the horny layer. 12 pieces of epidermis per kit are thus produced.

differentiate for 9 days in exposed culture. Finally, the epidermis consists of a mitotic layer, a spinous layer and a functional horny layer.

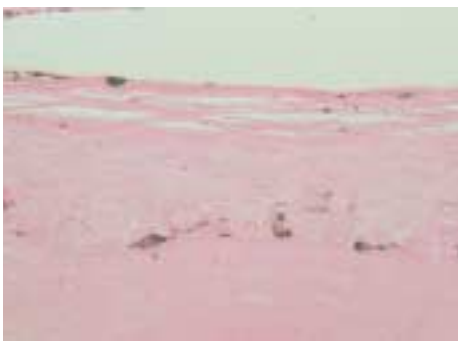
In March 1998, an *in vitro* validating study of cutaneous corrosion linked to 60 chemical products, carried out by ECVAM *(Europe Center for the Validation of Alternative Methods)* showed that Episkin™ allows good discrimination between chemical products with different structures and different corrosive potentials.

The Episkin™ kit is currently marketed in the form of 12 well plates. These kits are manufactured in the Gerland district of Lyons in a 2,200 m^2 skin bioengineering centre. During its first year of activity, more than 3,000 kits were produced and the production capacity allows for rapid growth.

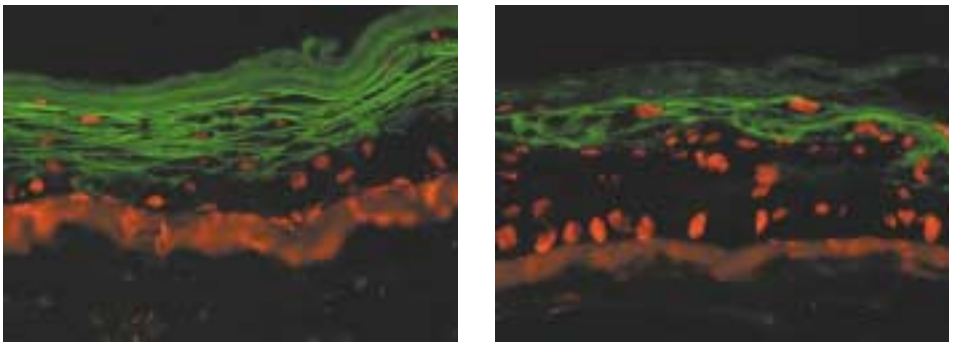
This kit is used to evaluate products *in vitro*, each of the 12 wells corresponding to an epidermal unit. Formulae to be tested are directly applied to the surface of the sample or in the culture medium. Analysis of results is made by measurements in the culture fluid, by cellular viability measurements and on histological sections of each epidermis.

Safety studies

Through the measurement of cellular viability and the concentration of defence mechanism markers, it is possible to demonstrate an irritation mechanism *in vitro*. The study of a series of cosmetic products on Episkin ™ has shown excellent agreement between the results of clinical tests and those obtained, *in vitro*, by 3 different industrial laboratories. In the course of the end 2003, the epidermis models validated for cutaneous corrosion are undergoing evaluation for cutaneous irritation.



The technique of co-culturing keratinocytes with the precursors of the Langerhans cells or melanocytes has made it possible to produce two other Episkin™ models, immunosensitive (*left*) and pigmented (*right*). At present, these two models have not been produced on an industrial scale and only exist in the laboratory.



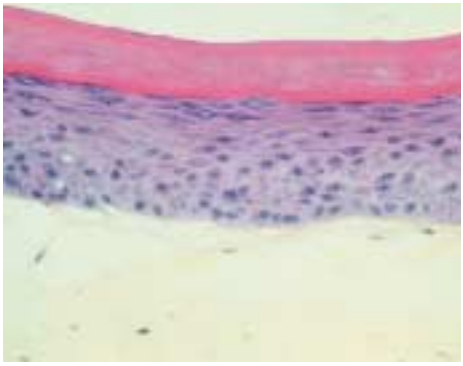
Episkin™ allows testing of retinol-based formulations by measuring the best tolerated concentration on keratinocyte proliferation *(right)*. The effect of this vitamin A precursor is also seen as a decrease in the differentiation of the keratinocytes, i.e. a thinner horny layer. *On the left*, a non-treated epidermis.

The advantage of these kits is that they allow *in situ* measurement of the effects of a substance applied to a human skin, experiments which are impossible directly in man, and thus permit selection of the best tolerated substance.

Tests of efficacy

Applied to Episkin™ vitamin C increases the production of essential lipids, the ceramides, in the horny layer. This shows that Episkin™ is a model where cellular metabolism is active. The protective effect of this vitamin against damage induced by UVA radiation has also been demonstrated and it produces an overall improvement in the barrier function of the epidermis. Formulations based on retinol, a vitamin A precursor, have also been studied on Episkin ™ in order to determine the best tolerated concentration which is most active on the proliferation of keratinocytes.

Episkin ™ is a first generation model of the epidermis. More complex second and third generation models already exist in the laboratory : they contain melanocytes and / or Langerhans cells. The introduction of the latter cells into the Episkin™ model by L'Oréal teams will be a decisive step towards the perfection of evaluation tests for contact allergy phenomena.



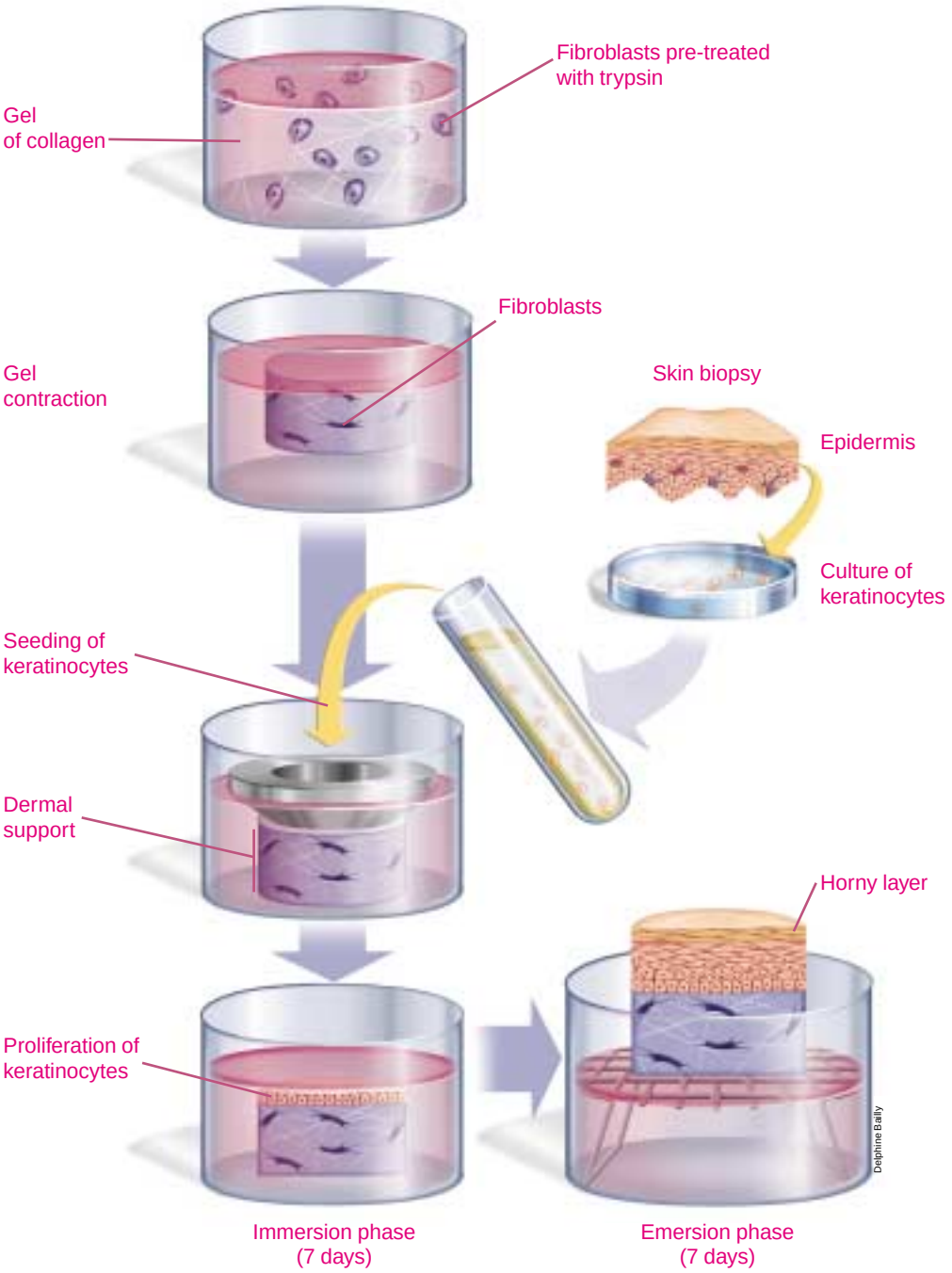
A model of human skin characterised by a living dermis topped by a functional epidermis has been perfected by L'Oréal laboratories. This model is a tool for scientists studying, among other things, the effects of ultraviolet A and B rays on the fibroblasts, the cells of the dermis.

MODELS OF HUMAN SKIN

The epidermis models previously described are reconstructed on inert dermal supports, which generally excludes their use for studying the physical properties of the dermis, the synthesis of the fibroblasts, the effects of UVA and UVB rays on these cells etc. In 1979 Eugene Bell, interested in the study of the ageing of fibroblasts in culture, had the idea of associating these cells with collagen in the same system. Once these two elements were brought together in an immersed culture he observed polymerisation of the collagen with the fibroblasts : the condensation of collagen microfibrils and the organisation of the fibroblasts caused a contraction of the gel which stabilised after a few days. A dermal equivalent was thus obtained and seemed to form the ideal support on the surface of which to reconstruct an epidermis.

The model with a living dermis

In 1986, in the L'Oréal laboratories, D. Asselineau and his team perfected a model of human skin : isolated keratinocytes were seeded onto the dermal support inspired by the Bell model. They divided for 7 days in immersion phase in a culture medium and it was all then exposed to air which allowed differentiation of the keratinocytes into corneocytes and thus formation of the horny layer. A functional epidermis had



To construct the Asselineau model, fibroblasts which have been treated with trypsin are mixed with collagen I. Organisation of the fibroblasts causes a contraction of the gel which is stabilised. After having seeded the keratinocytes in culture onto the dermal support, these cells proliferate for 7 days in an immersion phase and a final 7-day emersion phase causes the formation of the horny layer. A model of human skin (dermis + epidermis) is thus obtained.

©GUAILLARDE RAPHAEL / GAMMA PRESS

Children suffering from *Xeroderma Pigmentosum* (XP) must avoid any exposure of their skin to the sun : This genetic disease is characterised by the absence of DNA repair in cells damaged by radiation.

thus been reconstructed on a "living" dermis : a human skin equivalent was therefore achieved.

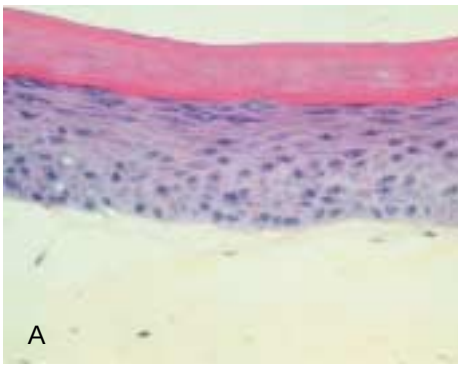
Using this model, F. Bernerd and D. Asselineau have studied major changes in skin morphology when it is subjected to UVB irradiation : a process of early apoptosis (programmed cell death) characteristic of sunburn and a decrease in the regulation of keratinocyte differentiation markers have been observed. Over a longer period, a proliferation of the keratinocytes allows a progressive regeneration of the epidermis.

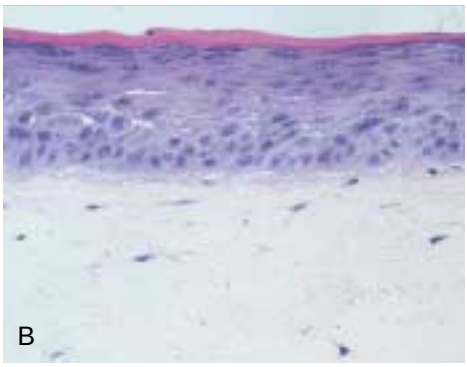
A year later, the same scientists described the effects of UVA radiation on the dermal compartment of the Asselineau model and the correlation with certain alterations observed in photo-aged skin : they observed the fibroblasts apoptosis 6 hours after exposure and the disappearance of most of these cells after 48 hours. Analysis of the regeneration of the dermis over the two weeks following showed that the surviving fibroblasts are activated and proliferate synthesising extracellular matrix proteins.

This was all knowledge of the physiological events involved in the effects of ultraviolet rays that the scientists have used to demonstrate the efficacy and photoprotector properties of Mexoryl® SX relative to both UVB and UVA rays.

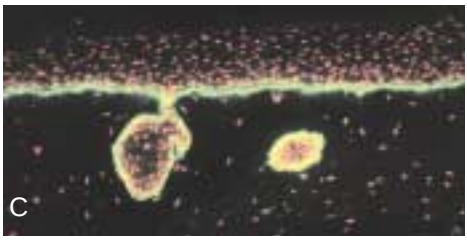
"Moon children's" skin recreated

Xeroderma Pigmentosum (XP) is a rare genetic disease characterised by an enzyme deficiency which prevents the repair of the cellular DNA. Children suffering from this disease, commonly known as "Moon





When a normal epidermis (A) and an *in vitro* reconstructed XP epidermis (B) are compared, the XP epidermis is characterised by a much thinner horny layer. After reconstructing an XP skin (dermis XP / epidermis XP), L'Oréal scientists observed, by immuno-labelling, invaginations of keratinocytes into the dermis in the XP equivalent dermis (C).



children", are excessively sensitive to sunlight, have accelerated cutaneous ageing and have a likelihood of developing skin cancers two thousand times higher than normal. F. Bernerd and D. Asselineau, in collaboration with the team of A. Sarasin at CNRS, have developed a reconstructed skin model with the characteristics of this deficiency. Keratinocytes and fibroblasts obtained from affected skin have allowed three models to be constructed : "Normal dermis / XP epidermis", "XP dermis / normal epidermis" and XP skin.

These models have already allowed essential observations to be made : the differentiation of the horny layer of the XP epidermis is greatly retarded and the fibroblasts do not have the same orientation in the dermis. Keratinocytes grown on a dermis containing XP fibroblasts also show a strong tendency to invaginate into the dermis, a phenomenon found in the early stages of cutaneous cancers. In addition, the reconstructed XP skins, subjected to exposure to UV radiation retain their lesion repairing deficiency. These are a great many results which open the way to a better understanding of this disease and early precancerous phenomena.

©GUAILLARDE RAPHAEL / GAMMA PRESS

In very serious burns, biomaterial dressings can be applied to the lesions. They protect the wound and allow time to reconstruct an epidermis from a biopsy made from an area of healthy skin. This epidermis reconstructed in the laboratory by cell culture techniques thus allows the dressing to be covered.

MODELS FOR MEDICAL APPLICATIONS

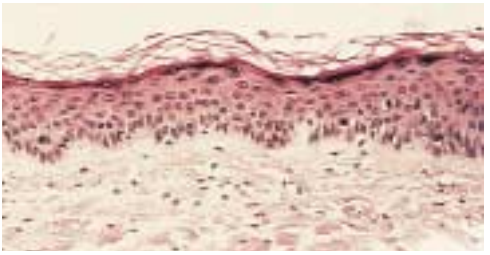
Models of the epidermis on an inert support, reconstructed human skin, cell cultures more and more suitable for including fundamental skin cells into these models, all these are advances in tissue engineering which have medical applications, in particular for serious burns and venous ulcerations.

Skin substitutes to aid major burns victims.

The extent, depth and location of the lesions are factors which determine the severity of a burn, and those which are deep second degree burns, where the superficial dermis is affected, require an auto-graft : skin taken from an intact area of the subject is grafted and provides definitive closure of the skin.

When the area to be grafted is too large, the burn victim's own skin is not available in sufficient quantity and an auto-graft is not possible. Temporary cutaneous cover with a skin graft from a donor is thus a technique which is widely used : the dermis causes fewer rejection phenomena and integrates into the remaining dermis. As far as the epidermis is concerned, it is rejected within a few weeks and will be replaced by an epidermis reconstructed in the laboratory from a biopsy of healthy skin from the subject, the keratinocytes of which have been cultured.

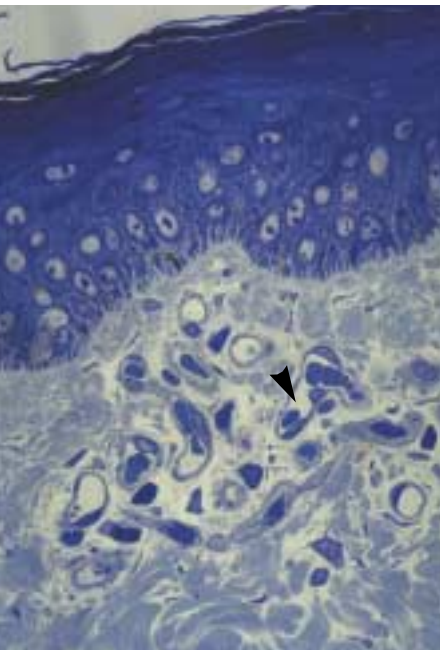
Tissue engineering technologies are being used and improved in the medical field and the systems are designed to meet specific requirements : the efficacy and nature of the dermal support, adhesion to the wound, the proliferation capacity of endogenous cells in the grafted matrix, the production of macromolecules which leads to good healing. In this respect, *Integra Artificial Skin* has been developed by two MIT research workers. *Integra* is a two-layer membrane system : the dermal equivalent consists of a porous matrix formed from collagen and a glycoaminoglycan (chondriotin 6 sulphate) and it is covered by a synthetic epidermal substitute (silicone). Placed on the wound, the dermal equivalent allows infiltration of fibroblasts, macrophages and lymphocytes from the wound and the temporary synthetic epidermis controls hydration. After sufficient vascularisation, the synthetic epidermis is replaced by the subjects own epidermal cells which proliferate on the neo-dermis to form finally a horny layer. A human skin with an epidermis and a dermis is thus reconstructed with the subject's cells. Already successfully tested on major burns patients, this system is also used for the treatment of cutaneous disorders caused by haemorrhagic necrosis.



Apligraf ® is a skin substitute formed from a collagen I gel seeded with fibroblasts and covered by an epidermis. *Apligraf* ® is intended for the treatment of leg ulcers of venous origin.

Apligraf ® for the treatment of venous ulcers

Apligraf ® is a skin substitute consisting of a dermis where the fibroblasts are collected from foreskins of newborn infants following circumcision. Seeded with keratinocytes, this dermal equivalent is finally covered by an epidermis with a horny layer. Apart from its role of covering and interactive dressing this skin substitute produces growth factors stimulating healing. Because of these properties, *Apligraf* ® is used for treating ulcers of various origins such as varicose ulcers, characterised by cutaneous lesions due to insufficient venous circulation in the lower limbs.



While the skins reconstructed in the laboratory are true human cutaneous tissue, there are many improvements to be made, in particular concerning vascularisation : today's models are a long way from including a network of blood capillaries, seen here *(arrow)* using optical microscopy on a skin biopsy.

MODELS FOR TOMORROW

The ideal skin substitute for treating major burns and ulcerations should have the following qualities : it should fulfil the major function of being a barrier against water loss and infection, have mechanical properties and strength equal to that of the skin ; it should allow rapid healing and be rapidly vascularised, with little immunological rejection, and it should be available in large quantities.

From the point of view of fundamental research, a better understanding of the skin requires the design of suitable tools and, in this respect, the valuable models already available are getting nearer and nearer the complexity of human skin.

Today, the challenge of biotechnology is to surpass laboratory standards wich accomodates itself with the variability of models at an industrial level, this will require models to be very reliable and reproducible.

Reconstructed skins are one of the main concerns of cosmetic industry, especially L'Oréal, and numerous basic scientists. Those models, technological breakthrough, were major medical advances, used for burn treatment (burn graft) and now to elucidate carcinogenesis mechanisms. Building a model, including nerve endings and capillary vessels is the new challenge researcher are trying to take up.

Cover : The skin, its different compartments and appendages as seen by Ludovic Hirschfeld in its « Traité et iconographie du système nerveux et des organes des sens de l'homme avec leur mode de préparation », 1866
Collection privée

L'ORÉAL

Direction Générale
Recherche et Développement

Direction de la Communication.
90, rue du Général Roguet, 92583 Clichy CEDEX France.
www.hair-science.com
www.skin-science.com